DECLARATION

It is herewith declared that this dissertation for the degree Master of Science (Bioanalytical chemistry) at the University of the Free State is the independent work of the undersigned and has not previously been submitted by him at any other University or Faculty for a degree. In addition, copyright of this dissertation is hereby ceded in favour of the University of the Free State.

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EFREM TECLEHAYMANOT ABAY DATE

Declaration certifying the candidate's personal contribution towards the research, which is the subject of this M. Sc. (Bioanalytical Chemistry).

Candidate: Mr. Efrem Teclehaymanot Abay (B. Sc. Hons)

Title: Development and Validation of Bioanalytical Assay Methods for Fentanyl in Human Plasma

Supervisor: **Prof. S.S. Basson**

Joint Supervisors: **Prof. H.K.L. Hundt**

Prof. W. Purcell

We, the undersigned, declare that under our supervision, Mr. Abay performed the development and validation of the assay methods contained in this dissertation. Under our supervision, Mr. Abay personally prepared and submitted full-length papers dealing with the assay methods described in this dissertation for publication in the Journal of Chromatography B. Mr. Abay personally compiled and typed the dissertation in its present form.

Prof. S.S. Basson	Prof. W. Purcell
Date	Date

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Development and Validation of Bioanalytical Assay Methods for Fentanyl in Human Plasma

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LIST OF ABBREVIATIONS

Abs	Absorbance
amu	Atomic Mass Units
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
CE	Collision Energy
CGMP	Current Good Manufacturing Practice
Cmax	Maximum Expected Concentration
CNS	Central Nervous System
Conc.	Concentration
Cpd	Compound
CV %	Coefficient of Variation
CXP	Collision Cell Exit Energy
DP	Declustering Potential
ECD	Electrochemical Detector
ELISA	Enzyme-linked Immunosorbent Assay
EP	Entrance Potential
ESI	Electrospray Ionization
FID	Flame Ionization Detector
FP	Focusing Potential
GC	Gas Chromatograph
GLC	Gas Liquid Chromatography
GLP	Good Laboratory Practice
Hrs	Hours

HPLC	High Performance Liquid Chromatography
i.d	Internal Diameter
ISTD	Internal Standard
i.v	Intra-venous
LC	Liquid Chromatography
LCQ	Liquid Chromatography with Quadruple MS
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantification
LLOD	Lower Limit of Detection
Min.	Minutes
MS	Mass Spectrometry
MS/MS	Mass Spectrometry/ Mass Spectrometry
NPD	Nitrogen Phosphorus Detector
% nom	Percentage of Nominal Concentration
QCs	Quality Control Standards
t_R	Retention Time
RPM	Revolution per Minute
RSD	Relative Standard Deviation
RIA	Radio-immuno Assay
Sec.	Seconds
SIM	Selective Ion Monitoring
Soln.	Solution
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
SPV	System Performance Verification
SPVS	System Performance Verification Standard

SRM	Selective Reaction Monitoring
STAB	Stability
STD	Calibration Standard
ULOQ	Upper Limit of Quantification
UV-Vis	Ultra Violet-Visible

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1 INTRODUCTION AND OBJECTIVES

1.1 Introduction

"Even today, the black bag carried by physicians would almost certainly contain an opioid analgesic, probably morphine sulphate. One hundred years ago, morphine would without question have been the most important drug in the bag; since there were no antibiotics, hormonal agents, or antipsychotic drugs, the practitioner depended heavily on drugs that would at least provide symptomatic relief" (Katzung 1987).

Compounds similar to morphine that produce pain relief and sedation have traditionally been called narcotic analgesics to distinguish them from the antipyretic analgesics such as aspirin and acetaminophen. However, the term "narcotic" is an imprecise one, since narcosis signifies a stuporous state whereas the opiates produce analgesia without loss of consciousness. The terms "opiate" and "opioid analgesic" are more appropriate, but established usage of a word is always difficult to extinguish. Consequently, "narcotic analgesics" are usually understood to include natural and semisynthetic alkaloid derivatives from opium as well as their synthetic surrogates with actions that mimic those of morphine (Katzung 1987).

The source of opium, the crude substance, and morphine, one of its purified constituents, is the opium poppy, *papaver somniferum*. The plant may have been used as much as 6000 years ago, and there are accounts of it in ancient Egyptian, Greek, Roman, and Chinese documents. It was not until the 18th century that the addiction liability of opium began to cause concern (Katzung 1987).

Opium contains more than 20 distinct alkaloids. In 1806, Serturner reported the isolation of a pure substance in opium that he named morphine, after Morpheus, the Greek god of dreams. The discovery of other alkaloids in opium quickly followed that of morphine (codeine by Robiquet in 1832, Papaverine by Merck in 1848). By the middle of the 19th century the use of pure alkaloids rather than crude opium preparations began to spread throughout the medical world (Goodman & Gilman 1985).

Large doses of opioid (narcotic) analgesics have been used to achieve general anaesthesia, particularly in patients undergoing cardiac surgery or other major surgery when circulatory reserve is minimal. Intravenous morphine, 1 mg/Kg, and subsequently the high-potency drug fentanyl, 50 µg/Kg, have been used in such situations with minimal evidence of circulatory deterioration. Despite such high doses, awareness during anaesthesia or postoperative recall has occurred. In addition, postoperative respiratory depression requiring assisted ventilation, may be a problem (Katzung 1987).

When large doses of fentanyl (50 to 100 µg/Kg) are administered by slow intravenous injection, profound analgesia and unconsciousness are induced. While this state is similar to that caused by morphine, the incidence of incomplete amnesia, hypotension, and hypertension is less than that associated with morphine; the duration of respiratory depression is also shorter (Kitahata and Collins, cited in Goodman & Gilman 1985). For these reasons, fentanyl has largely replaced morphine for anaesthesia, and it is utilized particularly during cardiac surgery, usually combined with muscle relaxants and nitrous oxide or small doses of other inhalation anaesthetics. Rigidity of respiratory muscles may be prominent during induction of anaesthesia with large doses of morphine or fentanyl, and administration of a muscle relaxant may be necessary to permit artificial ventilation (Goodman & Gilman 1985).

Following intravenous administration of fentanyl the onset of action is within one circulation time. The drug is rapidly redistributed, and the duration of action is approximately 30 minutes. However, accumulation of fentanyl occurs with repeated administration or following injection of large doses, leading to a prolonged duration of sedation and respiratory depression. Fentanyl is metabolized by the liver and is eliminated with a half-life of 3.5 hours (Goodman & Gilman 1985).

1.2 Objectives

The objective of this study was to develop a suitable, highly specific, and sensitive analytical method for the quantitation of fentanyl in the low nanogram range in human plasma.

2 METHOD DEVELOPMENT: CHEMICAL ASSAY

2.1 Introduction

The method development and establishment phase defines the chemical assay. The fundamental parameters for a bio-analytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined. Typical method development and establishment for a bio-analytical method include determination of (1) selectivity (2) accuracy, precision, recovery (3) calibration curve, and (4) stability of analyte in spiked samples (Guidance for Industry 2001).

2.2 Pre-development Literature Survey

Before method development is started, a comprehensive literature survey is made by the method developer to obtain as much information as possible about published assay methods for the drug to be assayed. The articles are carefully researched to decide on which method to establish and also for information about the stability of the analyte under various conditions.

2.3 Formulation of Analytical Plan

After performance of a thorough literature survey an analytical plan should be formulated. The summary of the literature survey should focus on the following points:

- Define validation, establish the need for validation, and identify significant validation parameters.
- Define and identify procedures for and summarize acceptance criteria for specific validation parameters.
- Define and identify procedures for and summarize acceptance criteria for secondary validation parameters and related topics (e.g. re-validation and system suitability). [The above three points are quoted from Jenke 1996].

The process of validating a method can not be separated from the actual development of the method, because the developer will not know whether the conditions for the method developed are acceptable until validation studies are performed. The development and validation of a new analytical method is therefore an interactive process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require re-validation. During each validation study, key method parameters are determined and then used for all subsequent validation steps. To minimize repetitious studies and ensure that the validation data are generated under conditions equivalent to the final procedure following a well-formulated sequential plan is required. The first step in the method development and validation cycle should be to set minimum requirements, which are essentially acceptance specifications for the method. A complete list of criteria should be agreed on by the developer and the end users before the method is developed so that expectations are clear (Green 1996). For example, is it critical that method precision (RSD) be 2 %? Does the method need to be accurate to within 2 % of the target concentration? Is it acceptable to have only one supplier of the HPLC column used in the analysis? During the actual studies and in the final validation report, these criteria will allow clear judgment about the acceptability of the analytical method.

2.4 Consideration of Analytical Variables

2.4.1 Matrix

Biological matrices exist as blood, plasma, serum, saliva, urine, tissues, skin samples, hair, seminal fluid etc. Some are plentiful and others scarce.

In this study of the determination of fentanyl concentration at sub-nanogram level human plasma is used as our biological matrix.

Important points to be considered are:

- Preparation of calibration standards and quality controls, i.e. introduction of the analyte into matrix.
- Extraction.
- Stability of analyte in matrix.

Different methods can be used to introduce the analyte into plasma, such as dissolving the solute directly in plasma, dissolving the analyte in a suitable solvent (water or organic solvent) and spiking the plasma with the solution. Since a small volume of plasma and a very low concentration of fentanyl are used during the analysis it is preferable to dissolve the analyte in a suitable solvent and spike the plasma with a small volume of the analyte solution; in the order of the spiking solution being less than 1 % of the biological fluid volume.

Extraction refers to the removal of the analyte introduced into the biological matrix. A simple one step extraction procedure is preferable to minimize the complex and time-consuming process. But sometimes using one-step Liquid-Liquid Extraction (LLE) gives impure extracts producing a lot of background noise in the chromatogram. Therefore a need for back-extraction or using Solid-Phase Extraction (SPE) arises. For many basic compounds Liquid-Liquid Extraction with a suitable organic solvent followed by back-extraction with a strong mineral acid and re-extraction from the acid, after alkalinisation, with a suitable organic solvent, even though time-consuming, is often found to be very effective in obtaining clean extracts for analysis.

Stability data is required to show that the concentration of analyte in the sample at the time of analysis corresponds to the concentration of analyte at the time of sampling. The stability of the analyte in analytical stock solutions, biological matreces, and processed samples (extracts) should be established. The stability of the analyte in a biological matrix should be conducted at the temperature, e.g. ambient and 4°C, and light levels that will be exprienced over the period needed to process a batch of study samples, and should include the effects of freeze-thaw, with a minimum of three cycles separated by at least 12 hours (Causon 1997).

2.4.2 Internal standard/external standard

The internal standard technique is very common in bio-analytical methodology especially with chromatographic procedures. The assumption for use of an internal standard is that partition characteristics of the analyte and the internal standard are very similar. This can be a

false assumption, and according to Curry and Whelpton (cited in Karnes *et al.* 1991) the only appropriate uses of nonisotopic analogue internal standards are to serve as qualitative markers, to monitor detector stability, and to correct for errors in dilution and pipetting (Karnes *et al.* 1991).

An important issue in method development is the use of internal versus external standardization. Quantification by external standard is the most straightforward approach since the peak response of the standard is compared to the peak response of the sample. The standard solution concentration should be close to that expected in the sample solution (Hewlett Packard 1994). Precise control of the injection volumes is mandatory because it influences the accuracy. Peak response is measured as either peak height or peak area.

For the internal standard method, a substance is added at the earliest possible point in the analytical scheme to compensate for sample losses during extraction, clean up, and final chromatography (Hewlett Packard 1994).

2.4.3 Detector

The choice of a detector depends on the chemical structure of the sample and the requirements of the method. There are various types of detectors, such as Ultra Violet-Visible (UV-Vis), fluorescence, Electro Chemical (EC), Mass Spectrometry (MS), and Mass Spectrometry/ Mass Spectrometry (MS/MS) in High Performance Liquid Chromatography (HPLC), and Nitrogen/Phosphorus selective detector (NPD), Electro Chemical (EC), Flame Ionization Detector (FID), MS, and MS/MS in the case of Gas Chromatography (GC). Depending on the physical and chemical properties of the drug and availability of the instrument, the most sensitive and selective detector should be used. For example, for samples containing a chromophore, UV-Vis; for trace analysis (1 ppm) fluorescence or electrochemical detection, etc. is preferable (SAVANT® 1992,1999).

2.4.4 Sample Preparation

To produce meaningful information, an analysis must be performed on a sample whose composition faithfully reflects that of the bulk of material from which it was taken (Skoog & West 1982).

Sometimes the sample preparation is a difficult problem, especially in clinical and environmental chemistry. General procedures are filtration (perhaps by means of a dedicated

membrane which retains compounds selectively), solid phase extraction with disposable cartridges (also with dedicated selectivity), protein precipitation, and desalting (Meyer 1979).

3 VALIDATION

The ultimate objective of the method validation process is to provide evidence that the method does what it is intended to do, reliably and reproducibly.

Method validation is a process for establishing that the performance characteristics of the analytical method are suitable for the intended application (Hewlett Packard 1994).

Results of a survey by Clarke (1994) on method validation of analytical procedures used in the testing of drug substances and finished products, of most major research based pharmaceutical companies with laboratories in the UK, indicated that although method validation shows an essential similarity in different laboratories there is much diversity in the detailed application of validation parameters. The greatest degree of consistency appears to be in the validation parameters applied to chromatographic procedures. According to Causon (1997), the key analytical parameters requiring validation include:

- Recovery
- Response function
- Sensitivity
- Precision
- Accuracy
- Selectivity
- Stability.

3.1 Pre-study Validation

3.1.1 Stability

Drug stability in biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solutions.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations (Guidance for Industry 2001).

3.1.1.1 Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles (Guidance for Industry 2001). At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen usually to about -20°C for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at −70°C during the three freeze and thaw cycles.

3.1.1.2 Short-term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

3.1.1.3 Long-term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study sample. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

3.1.1.4 Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

3.1.1.5 Post-preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. This is also commonly known as on-instrument stability.

3.1.2 Selectivity/Specificity

The terms selectivity and specificity are often used interchangeably. The term specific, however, refers to a method, which produces a response for only a single analyte. The term selective refers to a method, which provides responses for a number of chemical entities, which may or may not be distinguished. If the response for the compound of interest is distinguished from all other responses, the method is said to be specific (Hewlett Packard 1994).

Specificity can be established by comparing the chromatographic retention time of the analyte in extracted matrix samples, with its retention time in at least one reference solution or by mass spectrometric determination following chromatographic analyte separation. It can

also be investigated by analyzing at least six independent sources of the target matrix and checking for interference by endogenous matrix components. Any interference should be less than 20 % of the detector response at the Lower Limit of Quantification,LLOQ, (Causon 1997).

3.1.3 Accuracy, Precision, and Recovery

The *accuracy* of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of the actual value except at LLOQ, where it should not deviate by more than 20 %. The deviation of the mean from the true value serves as the measure of accuracy (Guidance for Industry 2001).

The *precision* of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20 % of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or reproducibility, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories (Guidance for Industry 2001).

The *recovery* of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100 %, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations

(low, medium, and high) with unextracted standards that represent 100 % recovery (Guidance for Industry 2001).

3.1.4 Procedure

The procedure for performing the validation must be presented in a complete, well-defined, practical and understandable format. Procedures should be outlined with sufficient detail so that all important experimental variables can be set to define values. While it is most advantageous for the procedures to be as broadly applicable as possible, exceptions should be clearly and completely stated (Jenke 1996).

3.2 Validation Process

3.2.1 Preparation of Calibration and Quality Control Standards in Biological Fluids

Calibration standards (STDs) will be prepared by spiking a pool of normal blank plasma with the stock solution (fentanyl citrate) to obtain 2 x expected highest concentration (2 x Cmax), which will be serially diluted (1:1) with normal plasma down to LLOQ standard. The calibration standards will be used to set up a calibration curve from which the concentrations of unknown samples can be calculated.

A matrix-based standard curve should consist of a minimum of five to eight standard points, excluding blanks (either single or replicate), covering the entire range (Shah *et al.* 2000).

Quality control (QC) standards will also be prepared by spiking a pool of normal blank plasma at concentrations of 1.8 Cmax, then serially diluting (1:1) down to the lowest QC standard, i.e. 1.2 – 1.3 x LLOQ standard. The importance of QCs is to monitor the performance of the assay procedure in achieving the expected level of accuracy and precision.

Calibration standards and QCs will be prepared by weighing plasma to avoid the use of volumetric equipment. This minimizes errors introduced by reading volumetric measurements, and thus increases precision and accuracy of the method.

3.2.2 Process of Validating the Assay Method

In validating the assay method over a specified concentration range, the intra- and inter-batch accuracy and precision will be calculated from 3 validation batches (one intra- and two inter-batch validations). In the intra-batch all the calibration standards will be analyzed in duplicates, while QCs will be analyzed in replicates of six. Results will then be quantified both with peak heights and peak areas, and the best quantification method will be used for the statistical analysis of the two inter-batch validations.

3.2.3 Preparation of a Typical Calibration Batch

Intra- and inter-batch validation run-sheets were prepared, in such a way that QCs, STAB samples, bench-top stability samples, freeze-thaw stability samples, SPVS, blank plasma, and Zero (blank plasma + ISTD), are evenly dispersed among the calibration standards.

Table-1: Intra-batch validation run-sheet

No	Sample	No	Sample	No	Sample	No	Sample	No	Sample
1	SYS 1	23	QC I	45	QC E	67	FT0.2Cmax 4	89	STD D
2	STD K	24	QC I (dil)	46	QC D	68	STD G	90	BLANK 6
3	STD K	25	QC H	47	QC C	69	STD G	91	STAB 6
4	BLANK 1	26	QC G	48	QC B	70	STD F	92	QC I
5	ZERO 1	27	QC F	49	QC A	71	STD F	93	QC I (dil)
6	STAB 1	28	QC E	50	FTCmax 3	72	BLANK 5	94	QC H
7	QC I	29	QC D	51	FT0.2Cmax 3	73	STAB 5	95	QC G
8	QC I (dil)	30	QC C	52	STD H	74	QC I	96	QC F
9	QC H	31	QC B	53	STD H	75	QC I (dil)	97	QC E
10	QC G	32	QC A	54	BLANK 4	76	QC H	98	QC D
11	QC F	33	FTCmax 2	55	STAB 4	77	QC G	99	QC C
12	QC E	34	FT0.2Cmax 2	56	QC I	78	QC F	100	QC B
13	QC D	35	STD I	57	QC I (dil)	79	QC E	101	QC A
14	QC C	36	STD I	58	QC H	80	QC D	102	STD C
15	QC B	37	BLANK 3	59	QC G	81	QC C	103	STD C
16	QC A	38	SYS 2	60	QC F	82	QC B	104	STD B
17	FTCmax 1	39	STAB 3	61	QC E	83	QC A	105	STD B
18	FT0.2Cmax 1	40	QC I	62	QC D	84	FTCmax 5	106	ZERO 2
19	STD J	41	QC I (dil)	63	QC C	85	FT0.2Cmax 5	107	SYS 3
20	STD J	42	QC H	64	QC B	86	STD E	108	STAB 7

21	BLANK 2	43	QC G	65	QC A	87	STD E	109	STAB 8
22	STAB 2	44	QC F	66	FTCmax 4	88	STD D		

The 3 SPVS will monitor the performance of the instrument through the run of the batch. The six blank plasma extracts placed after the calibration standards serve as indicators for any possible carry-over in the system and for selectivity/specificity purposes. The Zero samples will indicate if the ISTD contributes to the analyte`s response in the system. The stability samples (STAB) will show whether the analyte and ISTD are stable on the instrument, and the bench-top stability samples will show that they are stable at room temperature.

Intra-batch validation is followed by two inter-batch validations. In the inter-batch validation at least five levels of validation QCs from highest, medium, and low concentrations must be used. The concentration range will be:

Highest 1.9 x Cmax

High 0.8 x Cmax

Medium 0.5 x Cmax

Low 2.3 x LLOQ

LLOQ 1.2 – 1.8 x lowest calibration STD B

Table-2: Inter-batch 1 validation run-sheet

No	Sample	No	Sample	No	Sample	No	Sample
1	SYS 1	16	QC E	31	STAB 12	46	STD C
2	STD J	17	QC B	32	QC H	47	STD C
3	BLANK 1	18	QC A	33	QC G	48	BLANK 6
4	ZERO 1	19	STD H	34	QC E	49	STAB 14
5	STAB 9	20	BLANK 3	35	QC B	50	QC H
6	QC H	21	SYS 2	36	QC A	51	QC G
7	QC G	22	SATB 11	37	STD E	52	QC E
8	QC E	23	QC H	38	STD D	53	QC B
9	QC B	24	QC G	39	BLANK 5	54	QC A
10	QC A	25	QC E	40	STAB 13	55	STD B
11	STD I	26	QC B	41	QC H	56	STD B
12	BLANK 2	27	QC A	42	QC G	57	ZERO 2
13	STAB 10	28	STD G	43	QC E	58	SYS 3
14	QC H	29	STD F	44	QC B	59	STAB 15
15	QC G	30	BLANK 4	45	QC A	60	SATB 16

NB: In the inter-batch 2 validation run -sheet the STAB samples are replaced with Bench-top stability samples, the remaining is the same to table 2.

3.3 Batch Acceptance Criteria

Standards and QCs can be prepared from the same spiked stock solutions, provided the solution stability and accuracy have been verified. A single source of matrix may also be used, provided selectivity has been verified (Shah *et al.* 2000)

Standard curve samples can be positioned anywhere in the run. An example of standard curve sample position is at the beginning and end of the run. Blanks, QCs, and study samples can be arranged as considered appropriate within the batch (Shah *et al.* 2000).

Placement of standards and QCs within a run should be designed to detect assay drift over the run.

Matrix-based standard calibration samples: 75 %, or a minimum of six standards, when back-calculated (including ULOQ) should fall within ± 15 %, except for LLOQ, when it should be ± 20 % of the nominal value. Values falling outside these limits can be discarded provided they do not change the established model. Acceptance criteria for accuracy and precision as outlined in the section "specific recommendation for method validation" should be provided for both within and between batch experiments (Guidance for Industry 2001).

Quality control samples replicates (at least once) at a minimum of three concentrations [one within 3 x of the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end of the range (high QC)] should be incorporated into each run. The results of the QCs provide the basis of accepting or rejecting the run. At least 67 % (four out of six) of the QCs should be within 15 % of their respective nominal (theoretical) values; 33 % of the QCs (not all replicates at the same concentration) can be outside the ± 15% of the nominal value. A confidence interval approach yielding comparable accuracy and precision is an appropriate alternative (Guidance for Industry 2001).

The minimum number of samples (in multiples of three) should be at least 5 % of the number of unknown samples or six total QCs, whichever is greater.

Samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria. The data for rejected runs need not be documented, but

the fact that a run was rejected and the reason for failure should be recorded (Shah *et al.* 2000).

3.4 Documentation

The validity of an analytical method should be established and verified by laboratory studies, and documentation of successful completion of such studies should be provided in the assay validation report. General and specific SOPs and good record keeping are an essential part of a validation of an analytical method. The data generated for bioanalytical method establishment and the QCs should be documented and available for data audit and inspection (Guidance for Industry 2001).

4 FENTANYL: LITERATURE SURVEY

4.1 INTRODUCTION

Fentanyl, alfentanil, and sufentanil are increasingly used at present to provide relief from pain during anaesthesia in newborn infants although the methods available for pain measurement are limited. An analytical method with the sensitivity necessary to detect, quantitate, and separate these drugs at the therapeutic concentration is therefore extremely desirable. The widespread use of these potent drugs has created a need for chromatographic techniques to identify and quantitate low levels of these compounds in biological fluids. Due to the low level being monitored, the method of detection must be free of endogenous interference or external contamination (Bansal & Aranda 1995).

Fentanyl, N- (1-phenethyl-4-piperidyl) propionanilide (Figure-1) is a potent synthetic opiate commonly used for surgical analgesia and sedation. Fentanyl is approximately 200 times more potent than morphine, with a rapid onset (1 to 2 minutes) but short duration of action (30 to 60 minutes), and has minor cardiovascular effects but can induce respiratory depression, hypotension and coma. Because of its potency and quick onset, even a very small dose of fentanyl can lead to sudden death, its minimal lethal dose being estimated to be 2 mg (Baselt *et al.*, Hall *et al.*, Marchall *et al.*, and P.A.J. Janssen, cited in Choi *et al.* 2001).

Fentanyl and alfentanil are commonly used adjuncts or major anaesthetics in surgery. Despite greater equianalgesic respiratory depression, fentanyl is more often used postoperatively for pain management than alfentanil (Kumar *et al.* 1996). Fentanyl is used in high doses ("anaesthetic doses") for inducing loss of consciousness in patients undergoing cardiac surgery because of its wide safety margin and its ability to produce loss of consciousness with ablation of the stress response to surgery without causing cardiovascular depression

(Hall *et al.* and Janssen, cited in Fryirsa *et al.* 1997). It is also used in low doses for the treatment of severe pain ("analgesic doses") where it is found to have a rapid onset of action (Fryirsa *et al.* 1997).

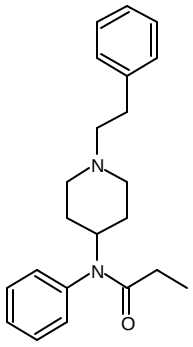


Figure -1: Chemical structure of fentanyl

Assay of the potent narcotic analgesic fentanyl demands high sensitivity, because the drug is effective in humans at plasma concentrations < 1 µg/L (Laganière *et al.* 1993). Because of the extremely low concentration of fentanyl in biological matrices its pharmacokinetic studies have proven difficult. The detection of lower levels of fentanyl from analgesic doses, however, is important for a full understanding of its pharmacokinetics (Bjorkman *et al.* cited in Fryirsa *et al.* 1997). A number of analytical methods capable of detecting these low concentrations have been developed. A High Performance Liquid Chromatography (HPLC) method with a lower limit of detection (LOD) of 1 ng/ml (Kumar *et al.* 1996), 0.12 ng/ml (Bansal & Aranda, 1995), and 0.15 ng/ml (Bansal & Aranda, 1996) were described. However, HPLC/UV methods lack sensitivity (Shou *et al.* 2001). Enzyme-Linked Immunosorbent Assay (ELISA) methods have also been utilized for detection of fentanyl with lowest detectable concentration of 100 pg/ml (Tobin *et al.* cited in Choi *et al.* 2001), but these methods have low selectivity and precision and are not suitable for pharmacokinetic studies. Radiochemical and Radio-immunoassay methods are rapid, sensitive and sufficiently selective, which require minimal amount of samples in the forensic laboratories for fentanyl screening, but suffer from a lack of selectivity at clinically realistic levels of fentanyl (< 10 ng/ml). This lack of selectivity may be partly responsible for the wide variability in kinetic parameters of fentanyl (Choi et al. 2001). Phipps *et al.* (1983) using RIA detected low plasma-fentanyl concentrations (LOD: 30 pg/ml), and Watts and Caplan (1990) reported calibration of the RIA in the range of 0.25 to 7.5 ng/ml to be linear. Generally immunoassays

tend to suffer from cross-interference (Shou *et al*. 2001). A number of Gas Chromatographic (GC) techniques have been reported. The Gas-Liquid Chromatography (GLC) method is rapid, simple, and reproducible, which has both selectivity and sensitivity to determine low concentrations of alfentanil (Chan 1988). However, selecting the appropriate extraction solvent is a problem. Complicated extraction solvents such as n-heptane-isoamyl alcohol, hexane-methanol, or toxic solvents like benzene require high temperature for evaporation, thus affecting analyte recovery and may result in unclear chromatograms with interfering peaks.

Gas chromatography coupled with mass spectrometry (GC/MS) is more specific and reliable for the detection of low concentration, (Shou *et al.* 2001). Fryirsa *et al.* (1997) devised a new fentanyl assay method optimized for high sensitivity and throughput of samples using GC/MS/SIM (Gas chromatography coupled with mass spectrometry using selected ion monitoring system). They used a one step extraction technique with sufentanil (Figure-2) as internal standard to give a high recovery from plasma, in the concentration range 0.02 to 25 ng/ml. The limit of detection, defined by a signal-to-noise ratio of greater than 3, was approximately 20 pg/ml.

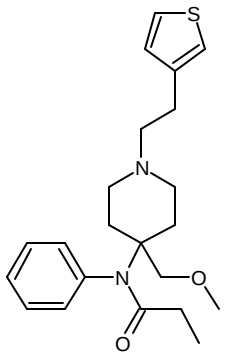


Figure -2: Chemical structure of sufentanil

Szeitz *et al.* (1996) reported GC/MS/SIM to be a selective and sensitive assay method (LLOQ: 0.05 ng/ml) for the quantitation of fentanyl in serum samples of swine, using a single step liquid-liquid extraction procedure.

Watts and Caplan (1988) used a GC/MS/SIM method to study fentanyl concentrations over the range 0.05 to 5.0 ng/ml, and found the overall recovery of fentanyl to be greater than 75 % over the range of 0.25 to 2.5 ng/ml. GC/MS offers the best sensitivity of the existing methods, but requires very long run times. LC/MS/MS has recently become the technique of choice for bio-analysis. Atmospheric Pressure Ionization (API) techniques namely Electron-

Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI), enable the generation of intact molecular ions ($[M+H]^+$ or $[M-H]^-$) from labile pharmaceutical compounds. The selected reaction-monitoring (SRM) mode of operation offers unparalleled specificity and this, in turn, allows minimal separation and very short LC run times, usually less than 3 minutes for a single analyte (Shou et al. 2001).

Particular effort has been made in the method development to automate the sample preparation step. As a direct result of the short analysis times offered by LC/MS/MS, sample preparation has become the rate determining step in the whole analytical cycle (Janiszewski *et al.* cited in Shou *et al.* 2001). With traditional solid phase extraction (SPE) or liquid-liquid extraction (LLE) preparation procedures, samples are processed manually in serial fashion. This process is labour-intensive and time-consuming. Therefore, much effort has been devoted to automating these processes. Although automated LLE and protein precipitation techniques have recently been reported, automated SPE using a 96-well plate format has enjoyed the greatest success and is the leading trend in industry for bio-analysis (Allanson *et al.*,Simpson *et al.*, cited in Shou *et al.* 2001).

4.1.1 Analyte Stability

Reports concerning the stability of fentanyl in biological matrices or stock solutions are presented in most of the literature; few among the many are summarized as follows:

Fentanyl citrate and sufentanil citrate (Janssen Pharmaceutica, Mississ, Ontario) in methanolic solutions are stable for 6 months at 4°C (Laganière *et al.* 1993). It states that major loss in drug recovery and decrease in assay precision are due to the adsorption of fentanyl to glass surfaces.

Björkman and Stanski (1988) performed stability tests of fentanyl and alfentanil simultaneously administered, 0.15-0.30 µg/min. Kg and 2.75-5.4 µg/min. Kg respectively, for 6 hours into three male Charles River F344 rats.

The rats were sacrificed and one kidney, half of the liver and a sample of abdominal wall muscle were frozen within 4 min., while the other half were wrapped in foil and kept on the bench for 1hr before freezing. The tissues were then stored at –20°C.

No significant differences in fentanyl and alfentanil concentrations between the organs that had been kept at room temperature for 1hr and the ones that had been frozen within 4 min. were observed.

They also tested the chemical stability of fentanyl and alfentanil. The stability of stock solutions of fentanyl and alfentanil in 10^{-3}M HCl with 10^{-6}M decylamine that had been kept in the refrigerator for 4 months, were compared to freshly prepared ones by the addition of sufentanil, extraction, and chromatography respectively.

In addition, 8 samples of fentanyl, alfentanil, and sufentanil in isopentanol (10 ng of each in 50 µL) were prepared, assayed by GC, left on the bench for 2 weeks and assayed again.

The concentrations of fentanyl and alfentanil in the stock solutions that had been kept refrigerated for 4 months were 101 and 103 %, respectively, compared to the concentrations in the freshly prepared solutions.

Keeping the isopentanol solutions of fentanyl, alfentanil and sufentanil at room temperature for 2 weeks changed the peak-area ratios fentanyl/sufentanil from 1.204 ± 0.024 to 1.165 ± 0.026 (mean (S.D, n=8), a 3.3% decrease ($p<0.02$) and the peak-area ratios alfentanil/sufentanil from 1.683 ± 0.044 to 1.660 ± 0.035, a 1.4 % decrease ($p>0.2$).

Addition of external standard to these solutions on day 0 or 14 showed that the absolute amounts of fentanyl, alfentanil, and sufentanil had decreased by less than 3 % over two weeks.

Fentanyl, alfentanil, and sufentanil are chemically stable and no breakdown of fentanyl or alfentanil was observed over 1hr in samples of liver and kidney, the two most important sites of drug metabolism.

Kumar *et al.* (1996) reported that frozen quality control samples of fentanyl and alfentanil in plasma tested over a 6 months period showed no sign of either degradation or loss. No significant differences was observed at all concentrations at times 0,1,2,3 and 6 months ($p>0.05$). Refrigerated solutions were injected daily at intermittent strengths to test stability. No major changes in peak area or height (i.e. 95 – 105 %) were observed over the time period of the study (6 months). Solutions adjusted with buffer to approximately pH 2.8 were injected at the beginning and end of each analytical sequence to assess the effect of low pH on stability within a given sample run. During a typical analysis of one subject's samples and standards (n = 30 – 35, 4.5 – 5.5 h) no alteration in peak height ratios or significant loss of individual peak heights or areas was discernible.

Shou *et al.* (2001) reported that analyte stabilities through multiple freeze -thaw cycles and on the bench at room temperature were tested by subjecting 6 replicates for each level of the regular QC samples (0.15,7.50, & 75.0 ng/ml) under these respective conditions, and then

extracting and analyzing them. The values obtained for these QC samples were then compared with their theoretical values. Stock solution stability was established by preparing a new sample of fentanyl and comparing the LC/MS/MS responses of secondary solutions (100 ng/ml in 1:1 acetonitrile/water) diluted from the new and the old samples. The stock solution was considered stable if less than 5 % difference in response was observed. The stability of fentanyl in frozen matrix was examined by freshly preparing a new set of calibration standards from the new sample and then analyzing the stability QCs (three replicates at each level of 0.15, 7.50, & 75.0 ng/ml) using the new calibration standards. Stability in re-constitution solvent was tested by re-injecting extracted samples (standard curve & triplicate QCs at each level) and comparing the results with those of freshly extracted samples.

Stability tests of fentanyl in stock solutions, in plasma, and in sample extracts were established. The fentanyl stock solution was stable at 4^{o}C for at least 147 days. The analyte was stable during storage, the sample extraction process, and LC/MS/MS analysis.

A summary of the features of assay procedures for fentanyl found during the literature survey is presented in Table-3.

Table-3: Features of published assay methods for fentanyl

Reference:	Method	Analytical Column	Extraction Method	LLOQ or LOD (ng/ml)	Comments
Bansal &. Aranda, 1995	HPLC-UV	Nova pack reverse phase cyano column 8mm x 100mm id, with 4um particle size (waters).	LLE with n-Hexane	LLOQ = 2.5 LOD = 0.12	Hydrolysis in acidic solution by cleavage to propionic acid decreases sensitivity.
Bansal &. Aranda, 1996	HPLC -UV	Waters 8mm x 100mm, 4um cyano column	LLE with acetonitrile & N-Hexane (1:6)	LLOQ = 0.15	The use of acidic pH 3 mobile phase suppressed the acidic silanol groups, allowing the elution of drugs.
Choi et. al, 2001	GC-NPD	HP-5, 5% phenyl-methyl siloxane (60m x 0.32mm,id, 0.25um film thickness).	LLE, with 5%isopropanol in n-butyl chloride (pH ~ 12).	LLOQ = 0.5 LOD=0.1	Extraction efficiency was high, b/c 1-high conc. of NaOH was used to denature plasma, since 80% of the fentanyl is bound to plasma protein. 2-addition of 5%isoprpanol to the solvent prevents adsorption of fentanyl to glassware.
Fryirsa et al., 1997	GC-MS	5%phenylmethyl silicone capillary column (HP-5Ms, Hewlett Packard) 30m x 0.25mm id, 0.25um film.	LLE with n-butyl chloride, pH ~ 12	LLOQ = 0.1 LOD = 0.02	D_5-fentanyl was added in high conc. Together with sufentanil (ISTD) and favourably competed with fentanyl for adsorption sites.
Gillespie et al., 1981	GC-NPD	2mx2mm,id, silanized glass column packed with 3% OV-17 on gas-chrom Q, 80%mesh	LLE with Hexane, back extracted with 1M HCl	LLOQ = 0.25 LOD = 0.1	1. Extracts should not be evaporated at high temp. (>50), since irreversible adsorption of drugs to the glass may occur. 2- Deactivation of the column by injection a plasma extract prior to the injection of sample extracts increase fentanyl sensitivity noticeably.
Kumar et al., 1996	HPLC-UV	Econosphere CN, 5um, 25cm x 4.6mm id, column.	LLE with Heptane-isoamyle alcohol (98:2).	LLOQ = 2 LOD = 0.25	Ionic strength of the back extractant was important & best results were obtained at 0.5M. Increasing it above 0.5M causes precipitation during analysis.
.Kumar et al., 1996	HPLC-UV	Spherisorb nitrile, 5um S5 CN column, 25cm x 4.6mm id.	LLE with Heptane	LLOQ = 1	
Laganière et al., 1993	GC-NPD	Ultra-2 capillary column (12.5m x 0.32mm,id, 0.5um film of 5% phenylmethyl silicone).	LLE with n-butyl chloride (pH 12), back extracted with 0.5M H_2SO_4	LLOQ = 0.25	A major loss in drug recovery & a decrease in assay precision are due to the adsorption of fentanyl to glass surfaces. So deactivation of reused glassware is vital.
Phipps et al., 1983	GLC-NPD	3.05m x 3.2mm silanized glass column with 3% OV – 17 on Gas Chrom Q (80 – 100 Mesh)	LLE with Benzene	LLOQ = 0.02	The use of organic solvents such as benzene produces a very broad deflection over the first few minutes of the chromatogram masking fentanyl peak at low concentration and giving superimposed peak at high concentrations. Therefore use water, which is relatively inert to organic solvents and inorganic substance, which is undetectable with NPD, for reconstitution purpose.
Portier et al., 1999	HPLC-UV	Spherisorb silica (5um, 250 x 4.6mm).	LLE with cyclohexane	LLOQ = 0.2	
Shou et al., 2001	LC/MS/MS	Beta silica column (50 x 3mm, 5um)	SPE with 2% NH_4OH in 80: 20 Chloroform /Isopropanol (v/v)	LLOQ = 0.05	
Stanski et al., 1988	GC-NPD	Fused-silica capillary column (25m x 0.31mm,id, with a cross-linked 5%phenylmethyl silicne).	LLE, with Iso-pentanol-pentane (1:49), pH~10, back extracted, with 0.1MHCl	LLOQ = 0.5 LOD = 0.1	Since the initial oven temperature was 100°C (i.e, 32°C below the B.Pt. of isopentanol), a solvent effect was conceivably present

Table continued on next page

Reference:	Method	Analytical Column	Extraction Method	LLOQ or LOD (ng/ml)	Comments
Szeitz et al., 1996	GC-MS	Hp-Ultra-2 cross-linked 5% phenyl-methyl silicone fused-silica capillary column (25m x 0.2mm,id, 0.33um film).	LLE with dichloromethane + TEA (0.5M)	LLOQ = 0.05	Sensitivity of the assay was increased by: 1- Using a low sample reconstituting volume (50ul). 2- Enhancing the chromatographic response of fentanyl (ca 1.5-2 fold) by placing a silanized glass wool plug in the injection port liner. 3- Adding TEA to the extraction & reconstituting solvents minimizes adsorption loses, and converts the residual citrate salt to freebase
Valaer et al., 1997	GC-MS	Fused-silica capillary column (15m x 0.2mm id, 0.33um film of 5%phenylmethyl-silicone gum phase, HP 5).	LLE with ethyl-acetate: n-butyl chloride (4:1), back extracted with 0.3N HCl	LLOQ = 0.3 LOD = 0.1	Derivatized with 0.1M pentafluoro benzyl chloride
Van Rooy et al., 1981	GC-MS	Capillary SCOT column 10m x 0.5mm id, with CARBOWAX 20M S.phase	LLE with benzene	LLOQ = 3.3 LOD = 3	Derivatized with 0.5ml acetic anhydride and 10ul pyridine
Watts & Caplan, 1988	GC-NPD & GC-MS	GC-NPD: 2 fused silica (0.32mm,id) capillary column 5% & 50% phenylmethyl-silcone GC-MS: 10m x 0.1mm,id, 0.34um 5%phenylmethyl-silcone.	LLE with N-chlorobutane (pH >10), back-extraction with 1N H_2SO_4	NPD LLOQ=0.1 LOD= 0.1 MS LLOQ = 0.05	While 100% recovery was seen using Hexane in Ethanol (19:1), the n-butyl-chloride extract (76% recovery) was found to produce the cleanest chromatogram with minimum background interferences.
Wattes & Caplan, 1990	GC-MS	Fused-silica (10m x 0.15mm,id) capillary column with 0.34um film of 5% phenyl methyl silicone	LLE with n-chlorobutane	LLOQ = 0.5	
Yuansheng et al., 1996.	GC-NPD	HP-crosslinked capillary wide-bore column (methyl siligum 10m x 0.53mm,id, 2.65um film).	LLE with cyclohexane-isopentanol (197:3) pH12 Back extracted with0.125mol/L H_2SO_4	LLOQ = 0.5 LOD = 0.2	Adsorption of drug onto the glass-ware decreases recovery.

4.2 Physical and Chemical Properties

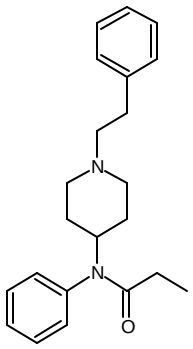
FENTANYL:

Synonym: Phentanyl

Chemical name: N-(1-phenethyl-4-piperidyl) propionanilide

Empirical formula: $C_{22}H_{28}N_2O = 336.5$

Structure:



Physical Characteristics: Crystals: Mp. 83°C to 84°C / Sparingly soluble in water (Moffat 1986).

FENTANYL CITRATE:

Proprietary Names: Fentanest; Leptanal; Sublimaze ^{R} / It is an ingredient of Hypnorm (vet.), Innovar, and Thalamonal.

Empirical formula: $C_{22}H_{28}N_2O.C_6H_8O_7 = 528.6$

Physical characteristics: White granules or a white glistening crystalline powder / Mp: 147°C to 152°C / Soluble 1 in 40 of water, 1 in 140 of ethanol, 1 in 350 of chloroform, and 1 in 10 of methanol; slightly soluble in ether (Moffat 1986).

Although fentanyl is a free base structurally related to pethidine, it is not a pethidine derivative. Unlike other narcotic types, the phenyl ring, which is attached to the piperidine nucleus through a nitrogen atom, is separated from the heterocyclic nitrogen by a chain of four atoms. Fentanyl is a weak base (pKa = 8.43). Solutions of fentanyl citrate are stable when stored at 4°C in well-closed, brown glass vials (Janssen, cited in Shipton 1983).

4.3 Pharmacokinetic Properties

4.3.1 Absorption

Most opioid analgesics are well absorbed from subcutaneous and intramuscular sites as well as from the mucosal surfaces of the nose and gastrointestinal tract. However, although absorption from the gastrointestinal tract may be rapid, the pharmacologic potency of some compounds taken by this route may be considerably less than after parenteral administration, because of significant first-pass metabolism in the liver after absorption. Therefore, the oral

dose required to elicit a therapeutic effect for such compounds may be considerably higher than that required when parenteral administration is used (Katzung 1987).

Fentanyl has a rapid onset and a short duration of action. In man, for example, a single intravenous dose of 500 to 1000 $\mu g.70kg^{-1}$ body weight immediately produces a pronounced state of surgical analgesia, respiratory depression, bradycardia, and other typical morphine-like effects. The duration of action is about 30 minutes. The rapid onset of action of fentanyl is related to the very rapid uptake of lipophilic drugs by the central nervous system. The short duration of action following a single intravenous dose of moderate size is due to its rapid elimination from plasma and brain as a result of extensive uptake of the unchanged drug by skeletal muscle and fat, and the rapid conversion of fentanyl to its metabolites. Accumulation of fentanyl in peripheral tissue compartments is extensive, because of the large mass of muscle and the high affinity of fentanyl for fat. Biotransformation is necessary for the ultimate excretion of the drug from the body. Biotransformation processes appear to be efficient, but the ultimate rate of fentanyl elimination may be limited by its rate of recirculation from muscle and fat to liver and kidney, where it is metabolized and excreted (Janssen, cited in Shipton 1983).

4.3.2 Distribution

The uptake of opiates by various organs and tissues is a function of both physiologic and chemical factors. Although all opiate analgesics bind to plasma proteins with varying degrees of affinity after absorption, the compounds rapidly leave the blood and localize in high concentrations in parenchymatous tissues such as lungs, liver, kidney, and spleen (Katzung 1987).

Fentanyl distribution in blood: volume of distribution is about 3 L/kg and distribution in blood plasma; whole blood ratio, 1.0. Protein binding in plasma is about 80 % (Moffat 1986).

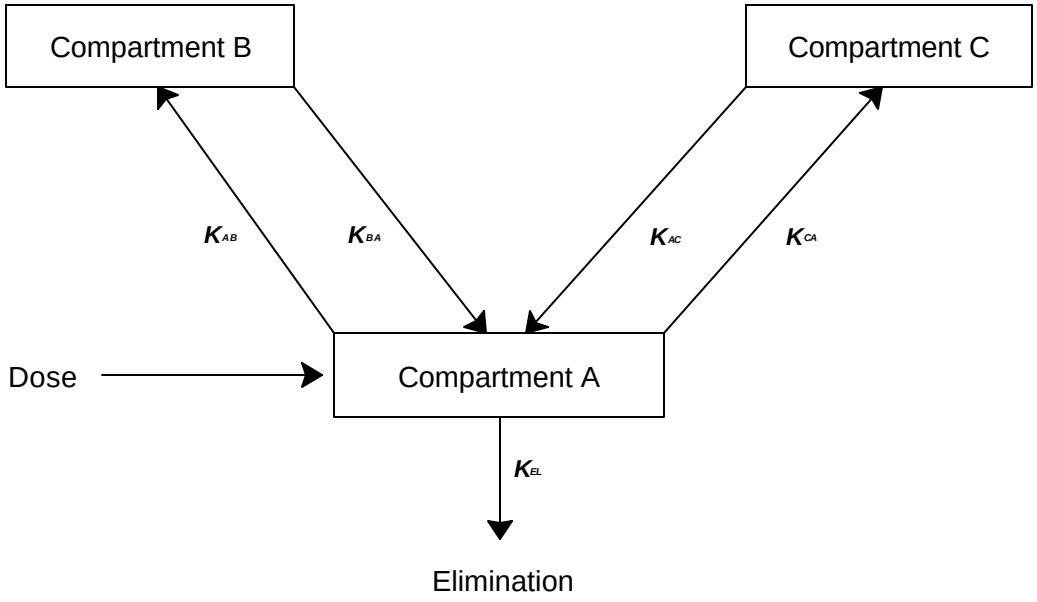
Although drug concentrations in skeletal muscle may be much lower, this tissue serves as the main reservoir for the drug because of its greater bulk. However, accumulation in fatty tissue can also become important, particularly after frequent high dose administration of highly lipophilic opiates that are slowly metabolized, such as fentanyl. Brain concentrations of opiate analgesics are usually relatively low in comparison to most other organs (Katzung 1987).

Björkman *et al.* (1990) determined the steady-state tissue/blood partition coefficients of fentanyl and alfentanil in 13 organs and tissues of a rat. A saline solution of fentanyl citrate, alfentanil hydrochloride, or both was infused over 6 h to achieve steady state at rates of 13 µg/kg.h for fentanyl and 120 µg/kg.h for alfentanil. Blood and tissue concentrations of drugs were measured by GLC (Gas Liquid Chromatograph). The partition coefficients of fentanyl were 2 to 30 fold higher than those of alfentanil. These data were then used in a physiologic pharmacokinetic model describing the disposition of the two opioids in humans. Compared with fentanyl, alfentanil is less lipophilic, has a smaller volume of distribution, a lower clearance, and a shorter terminal half-life. After bolus doses, the effects of both drugs are terminated by redistribution from the CNS to peripheral tissues. The computer simulations of the amounts of fentanyl or alfentanil in various organs and tissues in a 70 kg human after a bolus iv injection of 1 mg of either drug shows that the drug concentration peaks rapidly in the lungs, brain, heart, and kidneys. Because they are compartments where the ratio of apparent volume to blood flow is low, the capacity of the tissue for uptake of drug is small in relation to the rate of transfer of drug to the compartment. For the same reason, drug will also be removed rapidly from these compartments, which is reflected in the short mean transit time of the opioids in these tissues. The muscle and fat compartments represent the opposite. Large apparent volumes are filled with and emptied of drugs by means of limited blood flow. When the drug concentrations peak, muscle and fat together will contain approximately 30 % of the injected dose of fentanyl or alfentanil. The organs of the gastrointestinal tract fall into an intermediate category where filling and emptying of the compartments are moderately rapid. If simulation is stopped at 24 h after administering bolus doses of 400 and 5000 µg of fentanyl and alfentanil respectively, the terminal half-life of fentanyl can be estimated at 10 – 12 h, however, in the next phase it becomes 20 h. This last half-life is determined by the washout of drug from the fat and muscle compartments. Alfentanil on the other hand, attains a terminal half-life of 2.5 h after approximately 3 h. The brain concentration of fentanyl reaches a transient plateau at around 10 min after the injection, while for alfentanil the concentration peak increases sharply in less than 1 min. The washout of alfentanil from muscle and fat is also considerably faster than that of fentanyl, which to a large extent determines the respective elimination half-lives of the two opioids (Björkman *et al.* 1990).

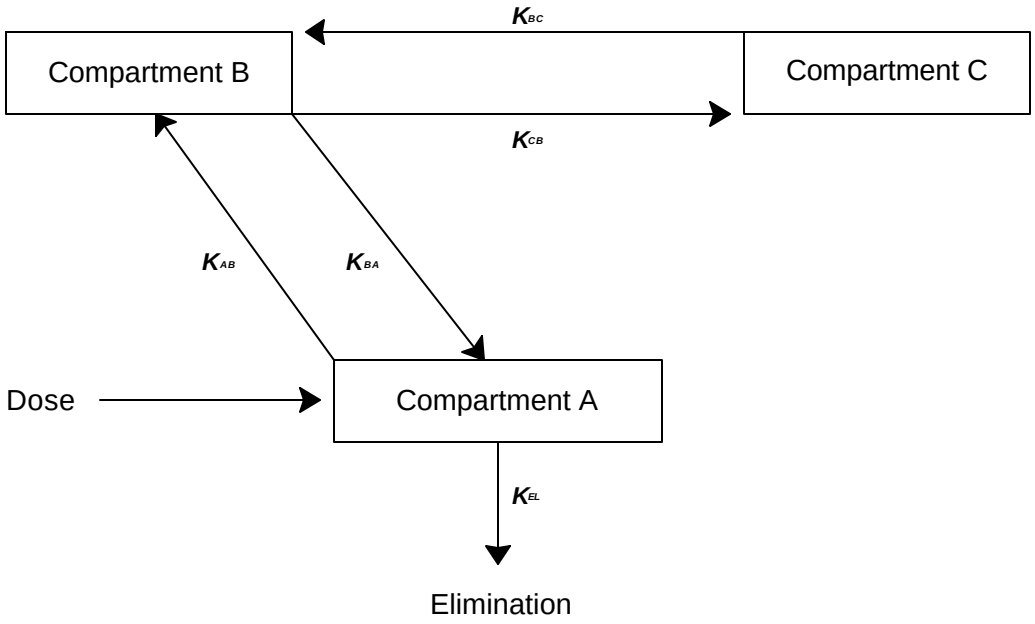
Hull and McLeod (1976) simulated the distribution and elimination of many drugs after iv injection by compartment mathematical models. After iv injection, the plasma concentrations of many drugs increases rapidly to a peak and then decay along a single or multi-exponential curve. They described the pharmacokinetics of fentanyl by a three-compartment model (type

A), in which the drug in the initial distribution volume (compartment A) redistributes to peripheral compartments B and C. Elimination is assumed to take place only from compartment A, and the plasma volume is considered to be part or all of compartment A.

Three Compartment Model (Type A)



Three Compartment Model (Type B)



In anaesthetic practice, regular increments of fentanyl are commonly given after an initial loading dose. Using the electrical analogue of the model, it is possible to predict the plasma concentrations, which might follow such a regime. It is also possible to determine the influence of the loading dose on subsequent plasma concentrations and to determine the degree to which accumulation might occur if increments were continued for extended periods (Hull and McLeod, 1976).

Hess *et al.* (1972), studied the tissue distribution of radiolabeled fentanyl in rabbits after intravenous administration. Fentanyl was rapidly eliminated from the plasma and concentrated in the organs and tissues. From his studies Hess found that fentanyl concentration in humans declines more slowly than in rabbits. The highest concentration of metabolites was reached after 20 min in rabbits and only after 3 hrs in man. This suggests that slower metabolism in man is responsible for the differences observed between the two species.

4.3.3 Metabolism

The opiates are converted in large part to polar metabolites, which are then readily excreted by the kidneys (Katzung 1987).

Fentanyl is rapidly metabolized, mainly in the liver (Janssen 1984).

Van Wyngaarden and Soudjin (cited in Shipton 1983) presented the metabolic pathways of fentanyl in the Wistar rat as shown in figure 3. Oxidative N-dealkylation and aromatic hydroxylation were found to be the major metabolic steps. Other workers have suggested an alternative metabolic path based on hydrolytic cleavage rather than oxidative N-dealkylation. None of the metabolites given in figure 3. have any analgesic activity, and thus it is likely that the analgesic effect is due to unaltered drug only.

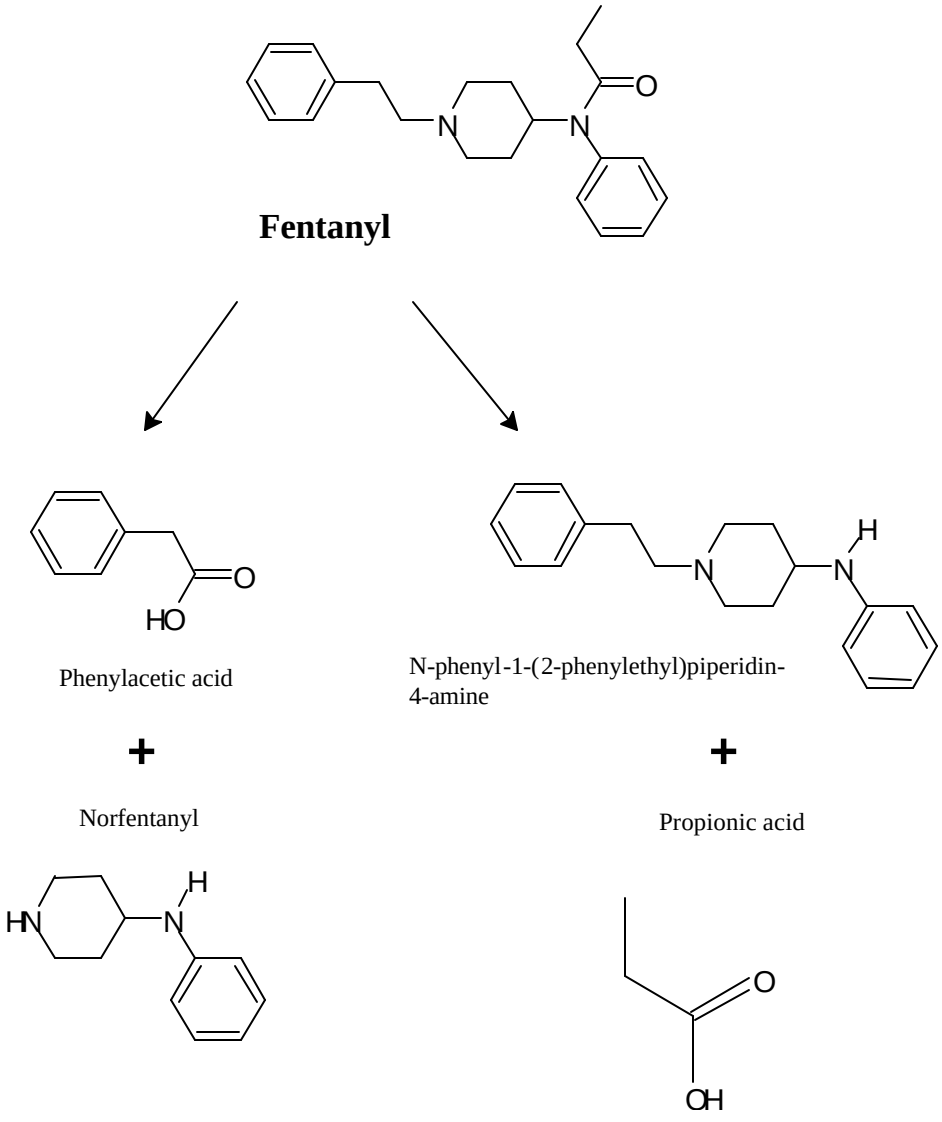


Figure -3: Metabolism of fentanyl

4.3.3.1 Half-life:

According to Hess *et al.* (1972), following an intravenous injection of 200 and 500 µg ^{3}H-fentanyl, the characteristics of the graph showing the total radioactivity of fentanyl in plasma during a 24 hrs period are similar with both doses. There is a rapid decrease in concentration during the first 10 – 20 minutes and then the plasma concentration of fentanyl declines only very slowly. The total radioactivity (fentanyl and metabolites) shows a tendency to rise smoothly, stays at a higher level until about 3 hrs, and then declines, too. The rate of diffusion from plasma to the tissue is high because 10 minutes after injection 98 % of the administered dose has already left the plasma. Within the first five minutes serum drug levels fall to about 20 % of the initial values and then decrease more slowly, with a half-life ($t_{1/2\alpha}$) of between 5 and 20 minutes (Schleimer *et al.*, cited in Shipton 1983). This is the distribution phase of the decay curve. The elimination phase is much slower with a half-life ($t_{1/2\beta}$) of ± 290 minutes. Bovill and Sebel (1980) described the kinetics of fentanyl following a bolus injection of 60 µg/kg by a bi-exponential decay curve, i.e. two compartmental model, with an initial half-life of 1.7 ± 0.85 min. and a half-life during the tissue uptake phase of 69 ± 8.2 min.

There was a highly significant decrease of 53 % ($p < 0.005$) in plasma concentration within 5 min. of the start of cardiopulmonary bypass. The reduction in plasma concentrations was greater than would have been expected from haemodilution alone (average decrease in haematocrit 41 %) and corresponds to fentanyl 8 ± 0.8 µg being redistributed to the tissue during the first 5 min. bypass. Plasma concentrations showed no significant change in the first 2 hrs following bypass, but thereafter declined exponentially with a mean half-life of 423 ± 36 min. The urinary excretion of fentanyl was small, the mean percentage of the original dose excreted up to 24 hrs was 2.1 ± 0.42 %. Analysis of the urine excretion rate, gave a mean elimination half-life of 354 ± 48.5 min., which was not significantly different from that calculated from the plasma concentration (Bovill & Sebel, 1980).

Bently *et al.* (cited in Shipton 1983) also showed a two compartmental model for the distribution and elimination of fentanyl with a $t_{½\alpha}$ of 1.83 minutes and a $t_{1/2\beta}$ of 230 minutes.

4.3.3.2 Elimination

The polar metabolites of the opiates are excreted mainly in the urine. Small amounts of the unchanged drug may also be excreted in the urine (Katzung 1987).

The more important pharmacokinetic and physiochemical properties of fentanyl and alfentanil are shown in table 4. Fentanyl is eliminated from the body almost exclusively by hepatic metabolism (McClain & Hug, cited in Stanski & Hug, 1982).

The liver is relatively efficient at metabolizing fentanyl since 60 - 80 % of the fentanyl is removed from the blood passing through the liver (first pass effect). Fentanyl`s extensive hepatic extraction results in a clearance of 10-15 $ml.kg^{-1}min^{-1}$, that approaches hepatic blood flow (18-21 ml/kg.min). Because the terminal elimination half-life of a drug is directly proportional to the volume of distribution and inversely proportional to clearance, fentanyl`s relatively long terminal elimination half-life is secondary to its large volume of distribution (l/kg) which results in low blood concentrations that limit the amount of fentanyl in the body delivered to and removed by the liver per unit time (Stanski & Hug 1982).

Because fentanyl has a relatively long terminal elimination half-life, its short duration of narcotic effect after a single dose can't be due only to its elimination from the body. As fentanyl is highly lipid soluble and readily traverses the blood-brain barrier, high brain concentration occur soon after a single intravenous injection and then decreases rapidly due to the redistribution of fentanyl to muscle and fat. After very large doses or multiple small doses of fentanyl, the redistribution mechanism of fentanyl becomes less effective in lowering the brain and plasma concentrations below the threshold for analgesia and respiratory depression (McClain & Hug, Murphy *et al* and Hug *et al.*, cited in Stanski *et al*, 1982). Under these circumstances, the duration of fentanyl`s narcotic effect is no longer short because the decline of blood and brain concentrations is dependent upon the slow elimination half-life. As the fentanyl dose is increased, the duration of the narcotic effect will also increase, and it becomes more difficult for the anaesthesiologist to judge the duration of fentanyl`s effect (Stanski & Hug, 1982).

Table-4: Pharmacokinetic and physiochemical properties of fentanyl and alfentanil

Property	Fentanyl	Alfentanil
Elimination half-life(h)	3.7±0.4	1.6±0.3
Clearance (ml/kg.min)	11.6±2.6	6.4±4.6
Volume of distribution at steady state (L/kg)	4.2±0.6	0.86±0.62
% of dose in urine unchanged drug	6.5	1% in dogs & rats
Metabolites (%)	69	75 in dogs & rats
Free fraction in plasma (%)	16	8
pK_a	8.43	6.5
% unionized at pH 7.4	9	89
Octanol:water partition coefficient at pH 7.4	860	130
Red blood cell/plasma concentration ratio	0.92	0.12

4.4 PHARMACODYNAMICS

4.4.1 Mechanism of action

The mechanism of action of fentanyl as an analgesic is not completely understood. It probably raises the pain threshold by acting on the thalamic and reticular areas of the brain. Furthermore, it has an effect on the cortex whereby patients experience a lack of concern regarding their pain.

5 GC/NPD ASSAY METHOD DEVELOPMENT

5.1 GC/NPD Assay Procedure

Introduction

In view of the scarcity and high cost of operational time on the GC/MS system a GC/NPD procedure was developed preliminarily to obtain information about the extraction process (purity of extracts and reproducibility of extraction), gas chromatographic parameters (oven temperature programs, injector temperature, column efficiency etc.) and to get an indication of the retention time of fentanyl under various conditions.

5.1.1 Preparation of Stock Solution

Materials

Considerable difficulties were encountered in obtaining pure fentanyl citrate reference compound. As it is not available in South Africa and had to be imported, a permit for its importation had to be obtained through the Medicines Control Council because it is classed as a narcotic. While waiting for the reference standard to arrive, it was decided to use fentanyl citrate injection solution (Sublimaze®), 50 µg/ml fentanyl free base equivalent, during the initial method development. Fentanyl citrate (Janssen Pharmaceutics), papaverine as internal standard, and high purity solvents (supplied from Burdick & Jackson) such as toluene, methanol, and ethyl ether were used.

Preparation of Injection Solutions

To prepare 10 µg/ml fentanyl stock solution in toluene, 1ml of fentanyl citrate injection solution (Sublimaze®), 50 µg/ml fentanyl free base equivalent, was transferred into a scintillation vial and its pH was adjusted to ~12 by adding a few drops of 5 M NaOH. Then 5 ml toluene was added and shaken vigorously for 5 minutes. The toluene phase was transferred to another tube and dried over about 1 g of anhydrous Na_2SO_4. The toluene extract was then decanted into a new screw-capped scintillation vial and stored in the refrigerator.

500 µg/ml papaverine.HCl solution in 0.01 M HCl (ISTD) was prepared by dissolving 6.14 mg papaverine in 12.28 ml of 0.01 M HCl. To obtain a solution which could be injected directly onto the GC column, a papaverine solution in toluene was prepared by dissolving 1 mg of papaverine in 100 ml toluene (i.e., 10 µg/ml papaverine in toluene).

5.1.2 Instrument and Chromatographic Conditions

Chromatography was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with an autosampler (HP 7673) and a Nitrogen/Phosphorus selective detector (NPD). A Chrompack CP-SIL 19 CB fused silica capillary column (30 m x 0.32 mm, i.d and 0.25 µm film thickness of 14 % cyanopropyl-phenyl, and 86 % dimethylsiloxane) was used with high purity helium (He) as carrier gas at a column head pressure of 25 p.s.i. and 4.6 ml/min flow at 150 °C.

GC conditions

Temperatures

Injector	285°C
Detector	310°C

Gas flow rates

He (carrier gas)	4.6 ml/min. at 150°C.
Detector Air	60 ml/min.
Detector H_2	3.0 ml/min.
Detector make-up N_2	44 ml/min.

Volume injected was 3 µl using splitless injection mode with a purge time delay of 1 min.

The initial oven temperature was 150°C for 1minute, and then it was programmed as follows:

Level	Rate(°C/min)	Final temp.(°C)	Final time(min)
1	30	270	2
2	5	280	9
		Total Run Time = 18 min.	

5.1.3 Chromatographic Results

For a fentanyl stock solution (10 µg/ml) a sharp peak was obtained at 10.551 min. with a signal-to-noise ratio of 100:1.

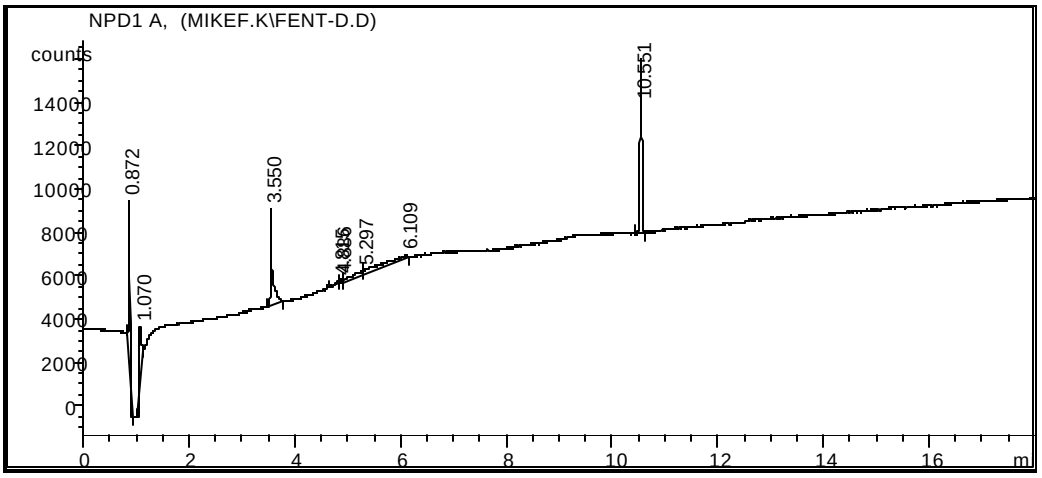


Figure -4: Chromatogram of 10mg/ml fentanyl in toluene

To obtain a solution with a S/N ratio closer to the expected concentration in final extracts the stock solution was diluted 5 times in toluene. The peak obtained at 10.334 min. had a S/N of 15:1.

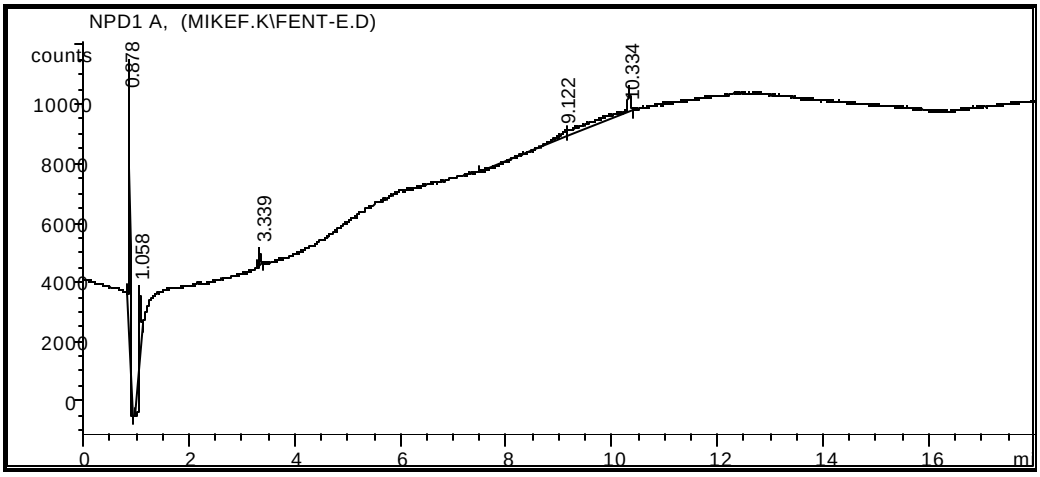


Figure -5: Chromatogram of 2 mg/ml fentanyl in toluene.

To optimise the total run time and retention time of fentanyl, five runs at different oven temperature programs were done.

Table -5: Summary of chromatographic runs at different oven temperature programs.

Injection # and split flow ratio	Initial Oven Temp (^{o}C)for 1min.	Rate (^{o}C/min.)	Final temp.(^{o}C)	Final time (min.)	Total run time (min.)	Retention time (min.)
1 (1:1)	200	20	280	5	10	8.84
2 (1:1)	250	10	280	5	9	6.815
3 (1:1)	280	Iso	280	8	8	4.976
4 (1:1)	260	20	300	3	6	4.873
5 (3:1)	260	20	300	3	6	4.712

The last run (#5) gave a reasonable run time and retention time and a good peak shape, thus for the following chromatographic runs this oven temperature program was used. Papaverine run under these conditions also yielded a peak at 5.8 min. with the same run time.

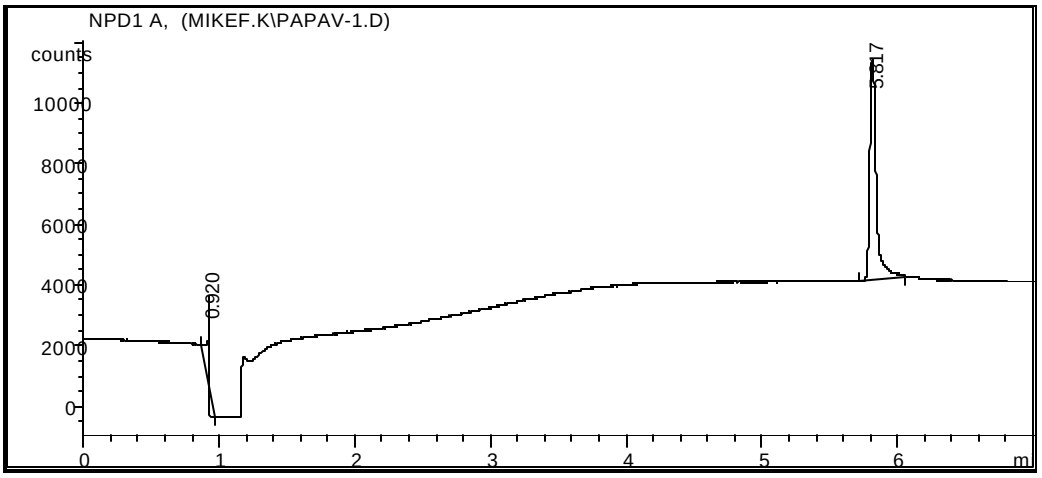


Figure-6: Chromatogram of 10 mg/ml papaverine in toluene.

5.1.4 Plasma Standards Sample Preparation

1ml of the 10 µg/ml fentanyl stock solution in toluene (previously prepared in section 5.1.1) was evaporated and the residue reconstituted in 200 µl methanol to obtain 50 µg/ml fentanyl in methanol. 6 ml Plasma was spiked with 120 µl of this solution to obtain a 1000 ng/ml fentanyl-plasma standard, which was further serially diluted 1:1 with blank plasma to obtain plasma samples with concentrations of 500, 250, 125, 62.5, and 31.25 ng/ml fentanyl. The dilution operations were not done volumetrically, but by weighing, taking into account that the specific gravity of plasma is 1.0269 kg/l. A summary of the sample preparation operations is presented in Table-6.

Table-6: Summary of preparation of fentanyl calibration standards

Code	Mass of Empty container(g)	Mass of cont. + blank fluid(g)	Mass of cont. + blank & spiked fluid (g).	Plasma fentanyl conc. (ng/ml)
Std-A	12.906	12.997	19.158	1000
Std-B	13.155	16.164	19.062	500
Std-C	13.114	16.164	19.152	250
Std-D	13.087	16.106	19.117	125
Std-E	13.156	16.161	19.155	62.5
Std-F	13.107	16.101	19.133	31.25

Extraction

The following extraction was performed on the plasma standards. Note that no attempt at extract clean-up (e.g. back extraction) was made at this stage. The reason for not doing any clean-up at this stage was to compare the chromatograms obtained with NP selective detection with those of SIM detection at a later stage.

- Pipette aliquots (1 ml) of the standards (A to F) into 5 ml disposable glass ampoules
- Add 100 µl of 1 µg/ml papaverine solution (ISTD prepared by diluting 20 µl of 500 µg/ml papaverine solution to 10 ml in 0.01 M HCl).
- Add 100 µl of 4 M NaOH and 2 ml ethyl ether
- Vortex for 2 min.
- Centrifuge at 2000 rpm for 5 min.
- Freeze aqueous phase in a freezing bath at ~ - 20 °C
- Decant organic layer into another ampoule and evaporate it at 40°C under a N_2 stream
- Reconstitute the residue in 100 µl of toluene
- Transfer to a 200 µl autosampler injection vial and inject 3 µl onto the GC-column.

A summary of the results obtained with the range of calibration standards A-F are presented in Table 7 below:

Table -7: Fentanyl calibration standard data

Code	Fentanyl concentration (ng/ml)	R.T for fentanyl (min.)	R.T for ISTD (min.)	Fentanyl peak area	ISTD peak area	Peak area ratio
Std-A	1000	4.711	5.952	65222	9004	7.244
Std-B	500	4.600	5.817	37888.6	10772	3.517
Std-C	250	4.575	5.787	15551	12443	1.249
Std-D	125	4.571	5.780	6488.3	11282	0.575
Std-E	62.5	4.578	5.792	4769.3	12739	0.374
Std-F	31.25	4.580	5.790	3177.8	13973	0.227

The signal-to-noise (S/N) ratio for 31.25 ng/ml is 13.

A full chromatogram of the STD B extract and expanded relevant sections of three chromatograms (STDs B, D and F) are presented below.

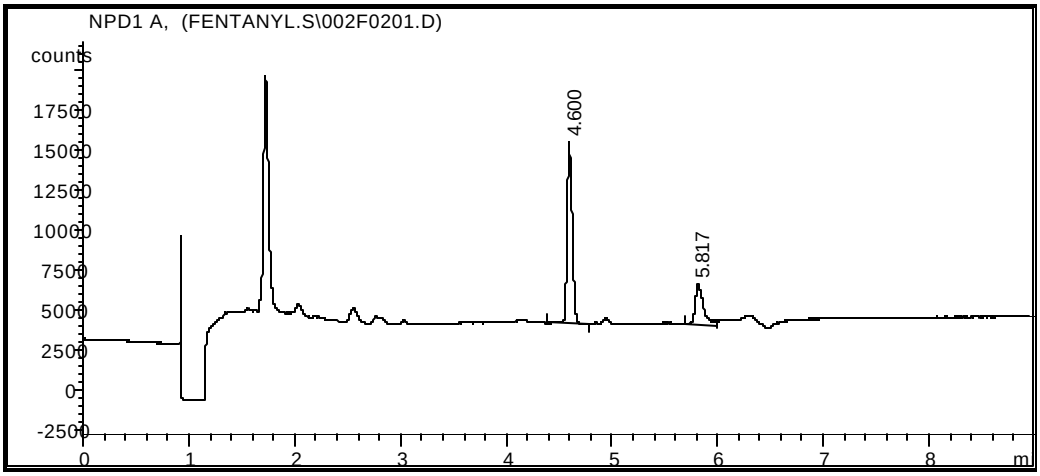


Figure -7: Full chromatogram of a 500 ng/ml plasma-fentanyl extract (STD B)

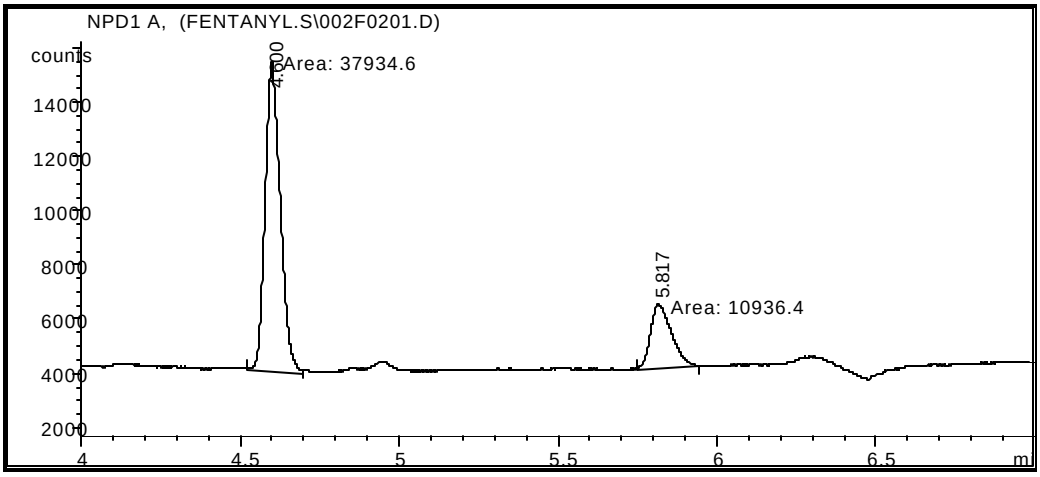


Figure -8: Chromatogram of a 500 ng/ml plasma-fentanyl extract (STD B)

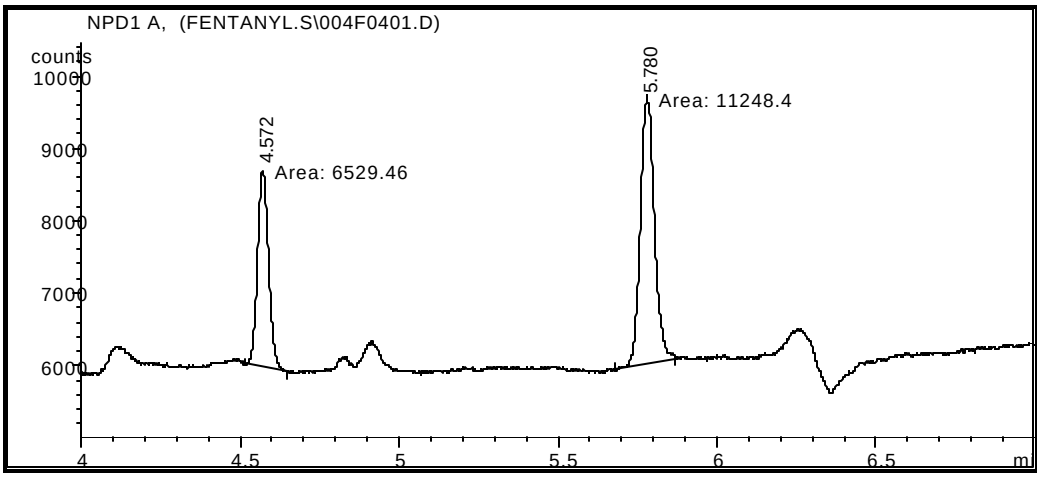


Figure -9: Chromatogram of 125 ng/ml plasma-fentanyl extract (STD D)

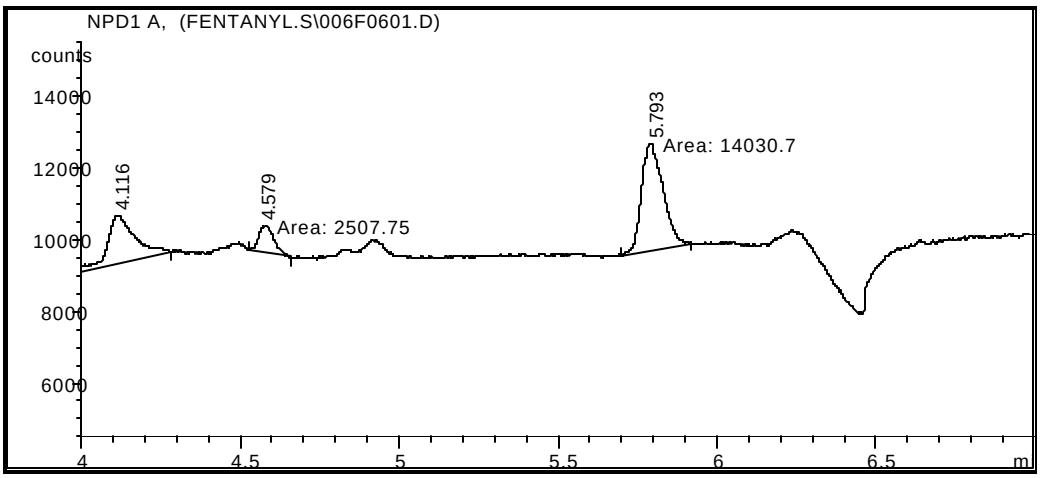


Figure -10: Chromatogram of 31.25 ng/ml fentanyl -plasma extract (STD F)

A calibration curve constructed with the data in Table-7 is presented in Figure-11.

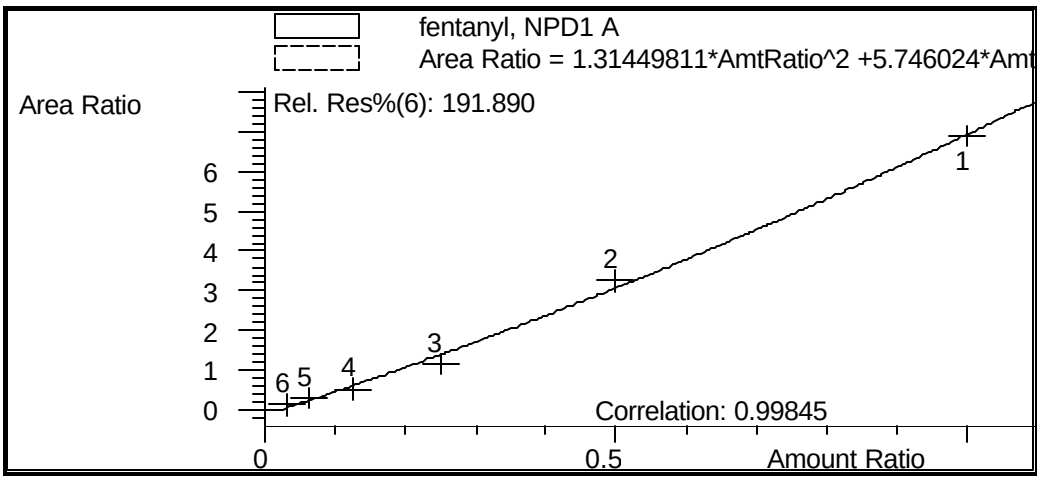


Figure -11: Calibration curve of fentanyl

The data fitted a second order regression equation $y = aX^2 + bX + C$ with a correlation coefficient R= 0.99845.

The chromatograms obtained and the calibration data of these plasma extracts demonstrated that it would be feasible to develop a GC/NPD assay method using a very simple extraction procedure. However, the cost-saving of a simple extraction procedure would have to be weighed up against the long-term effect of injecting such "dirty" extracts onto an expensive capillary column.

At this stage it was decided to start with the development of a GC/MS assay procedure described in section 9.1.

5.1.5 GC/NPD Assay Development using Extensive Sample Clean-up

Experience with the preliminary development of the GC/MS assay procedure described in section 9.1 indicated that better sample clean-up was imperative for an assay method of fentanyl to be successfully validated for the analysis of a large number of plasma samples. It became clear that the injection of a large number of relatively "dirty" extracts had a deleterious effect on the MS detector performance. It was therefore decided to develop an extraction procedure that would yield much cleaner extracts.

Extraction procedure

Back-extraction procedures are relatively well-standardised procedures to obtain clean extracts from biological fluids when the analyte of interest is either a basic or an acidic compound. In the case of a basic analyte the process involves the alkalinisation of the biological fluid followed by liquid/liquid extraction with a suitable organic solvent. The basic components in the organic phase are back-extracted into a strong mineral acid followed by re-extraction from the acid, after alkalinisation, with a suitable organic solvent which is then evaporated to yield the final extract which is then reconstituted in a small volume of an organic solvent suitable for injection onto gas chromatography columns.

The following double back-extraction procedure was therefore developed to obtain clean extracts of fentanyl and the internal standard sufentanil from plasma:

- Pipette 1 ml plasma into a 10 ml disposable glass ampoule
- Add 10 µl of the 5 µg/ml sufentanil solution for injection
- Add 50 µl of 10 M NaOH and 5 ml ethyl ether
- Vortex for 2 min.
- Centrifuge at 2000 rpm for 5 min.
- Freeze aqueous phase in an alcohol freezing bath at ~ - 20 °C
- Decant organic layer into another disposable glass ampoule containing 1.5 ml 1N H_2SO_4
- Add 0.5 ml 10 M NaOH and 5 ml ether
- Vortex mix vigorously for 2 minutes
- Centrifuge at 2000 rpm for 5 minutes
- Freeze aqueous phase in an alcohol freezing bath at ~ - 20 °C
- Decant the ether phase into a 5 ml disposable glass ampoule.
- Evaporate ether under a stream of nitrogen
- Reconstitute the extract in 100 µl toluene
- Transfer the final extract to a 200 µl autosampler vial
- Inject 3 µl onto the GC column

5.1.6 Instrument and Chromatographic Conditions

A chrompack CP-SIL 8 CB fused capillary column (30 m x 0.32 mm i.d. 0.25 µm film thickness of 5 % phenyl / 95 % dimethyl polysiloxane), using high purity helium carrier gas at 25 psi was used in the GC/NPD set up described in section 5.1.2.

GC Conditions

Injector and detector temperatures were as described in section 5.1.2.

The initial oven temperature was 80ºC for 1minute, and then was programmed at a rate of 30°C/min. to 300°C where it remained for 3 minutes before cooling down to 80°C. The total chromatography run time was 11.33 min.

Under these conditions fentanyl was eluted at a t_R = 9.330 min. and sufentanil at t_R = 9.639 min.

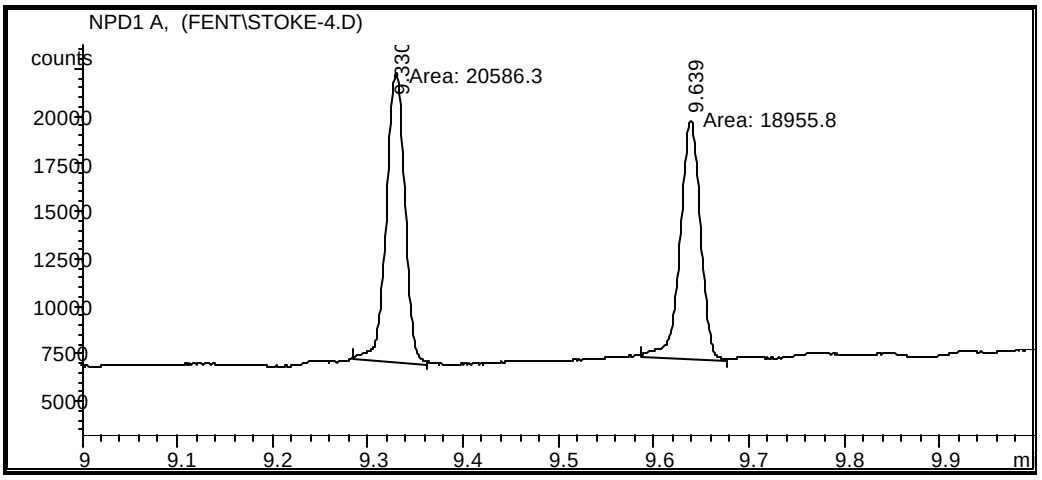


Figure -12: Chromatogram of fentanyl t_R = 9.33 min. and sufentanil t_R = 9.64 min.

The extent of the extract clean-up is illustrated by the following two chromatograms.

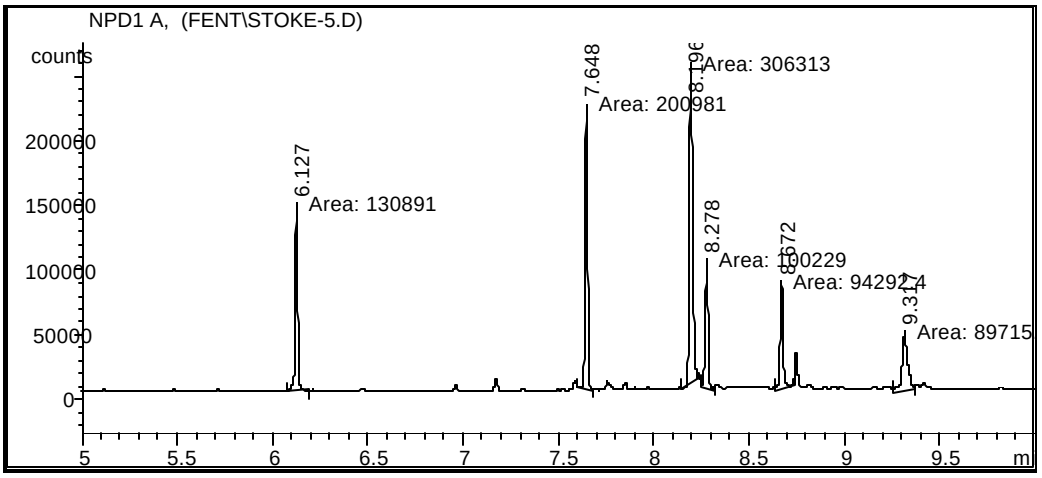


Figure -13: Chromatogram of single ether extract of blank plasma spiked with 500 ng/ml fentanyl, t_R = 9.31 min.

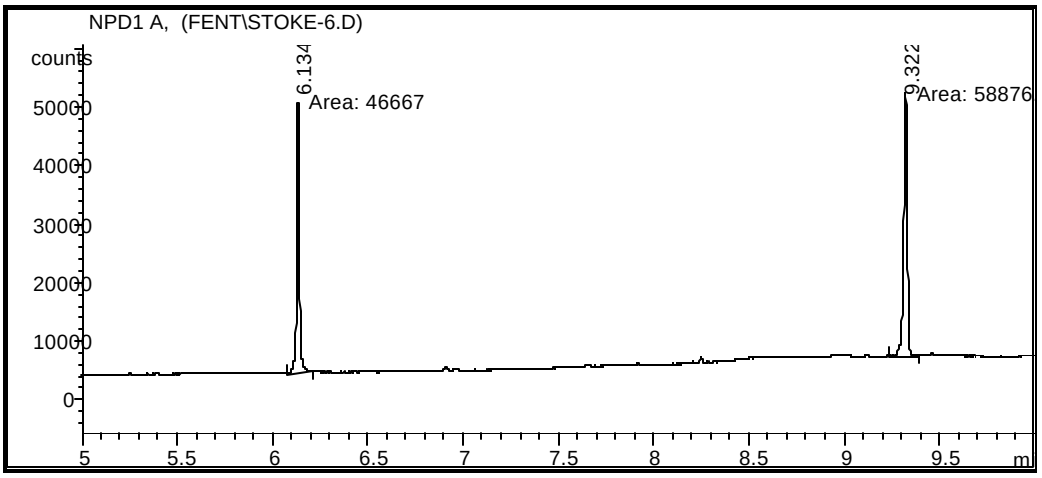


Figure -14: Chromatogram of double back-extracted ether extract of plasma spiked with 500 ng/ml fentanyl, t_R= 9.32 min.

The chromatogram of the double back-extracted plasma extract contains only the fentanyl peak (t_R = 9.322 min.) and one additional peak with a t_R = 6.134 min. while the extraction yield for fentanyl has decreased to 58876/89715 x 100 = 66 % relative to the extraction yield of the single ether extraction. However, the lower extraction yield is more than compensated for by a trouble-free assay method when a large number of samples need to be assayed.

5.1.7 Preparation of Calibration Standards

10 ml Blank human normal plasma was spiked with 20 µl of fentanyl citrate injection solution to obtain 100 ng/ml fentanyl plasma concentration. Then the plasma stock solution was serially diluted 1:1 with blank plasma to produce 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 ng/ml plasma-fentanyl concentrations.

Aliquots (1 ml) of the above standard plasma solutions were pipetted into 10 ml disposable glass ampoules, containing 10 µl internal standard solution (sufentanil injection solution, 5 µg/ml free base) and the double back-extraction performed on each. Extracts were then reconstituted in 100 µl of toluene and 3 µl injected onto the GC column.

A summary of the results is presented in Table-8.

Table-8: Fentanyl calibration standard data.

		Peak Area			
Code	**Conc.(ng/ml)**	**Fentanyl**	**Sufentanil**	**Ratio**	**S/N**
STD A	100	55211.6	21882	2.523	
STD B	50	28728.9	24699.4	1.163	
STD C	25	15323	28069.9	0.546	
STD D	12.5	8230.7	29885.3	0.275	
STD E	6.25	3499	22826.7	0.153	14
STD F	3.125	1940	25108.2	0.077	18
STD G	1.56	1059	31592.2	0.034	9
STD H	0.78	959	34733.7	0.028	9

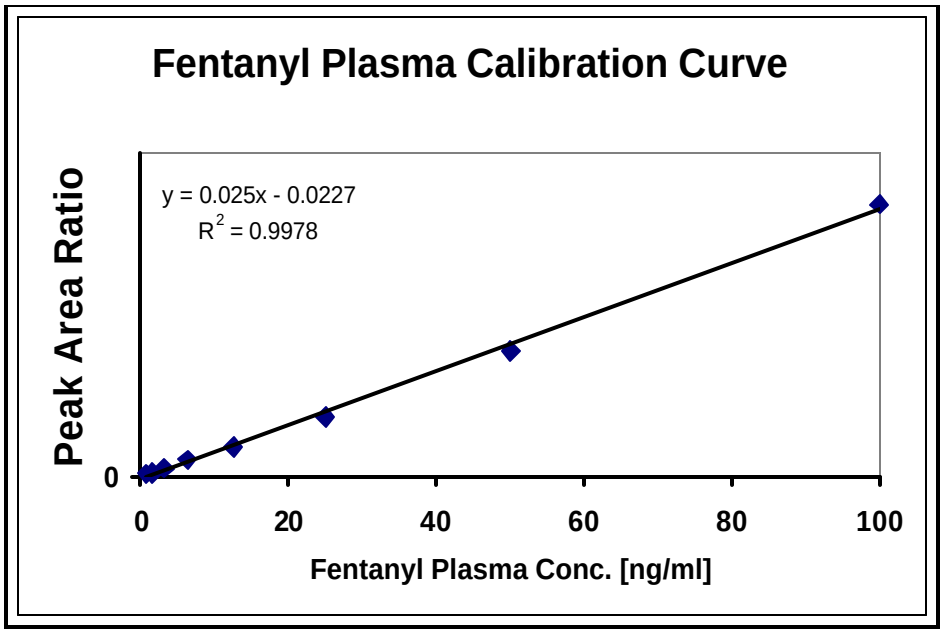


Figure -15: Linear calibration line

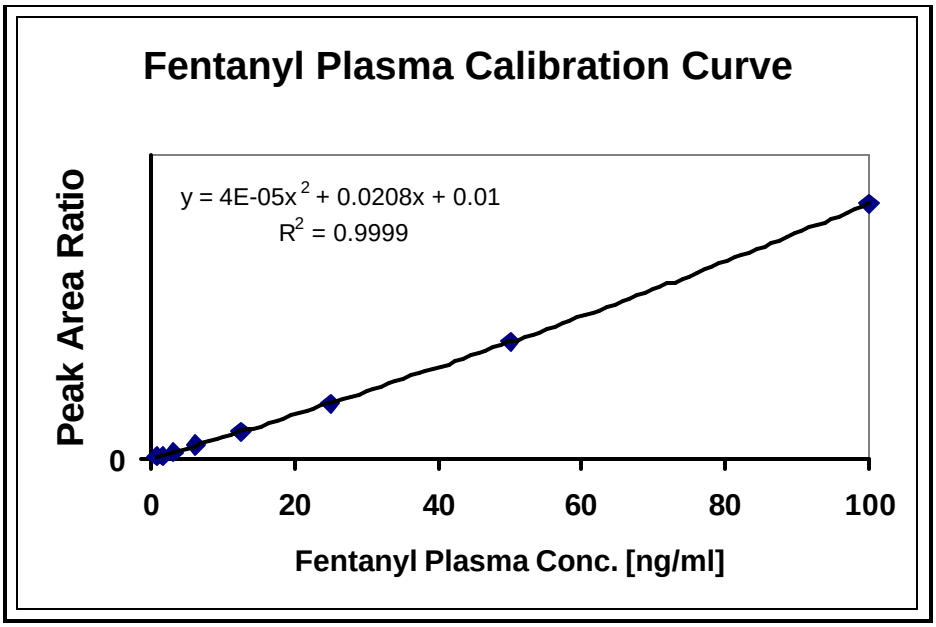


Figure -16: Quadratic calibration line

As can be seen from Figures-15 and -16, a second order regression fits the calibration data better than a linear regression.

Optimising the turn-around time

The assay method performed very well but a disadvantage of the assay method at this stage, was the rather long turn-around time of 19.5 minutes. The long turn-around time was partly due to the long chromatography time, as well as the time it took the oven to cool down and equilibrate again to 80°C accounted for about 7 minutes of this time. The most efficient way of reducing the turn-around time is to increase the initial oven temperature as high as possible without losing too much column efficiency and resolution due to band spreading during sample injection and to ramp the oven temperature at as high a rate as feasible. This reduces the chromatography time as well as the oven cooling down and equilibration phase time. The results of several chromatographic runs at various initial oven temperatures and oven temperature ramp rates are summarised in Table-9.

Table-9: Summary of chromatographic data for runs at various initial oven temperatures and oven temperature ramp rates

		Splitless injection		Split Injection			
IOT (^oC)	**OTRR (^oC/min)**	**Rt(min.)**	**Peak Area**	**Split ratio**	**Rt(min.)**	**Peak Area**	**TAT (min.)**
80 for 1min.	30	9.23	29319	8 to 1	9.21	24916	19.5
140 for 1min.	40	6.20	21904	10 to 1	6.19	26741	12.0
200 for 1min.	40	4.62	26312	13 to 1	4.69	23974	8.5
240 for 1min.	40	3.45	20088	15 to 1	3.47	21398	6.5

Notes: **IOT** = Initial oven temperature, **OTRR** = Oven temperature ramp rate, **TAT** = Turn-around time
Final temperature and hold time = 300 °C for 2 minutes

From Table-9 it is clear that the turn-around time can be reduced significantly, thereby increasing the sample throughput without significant changes in the performance of the assay method. However, an initial oven temperature of 240°C would obviously be too high as indicated by the deterioration of the peak shape (see Figure-19). The following chromatograms illustrate the effect of some of the temperature programs on the peak shapes of the analytes.

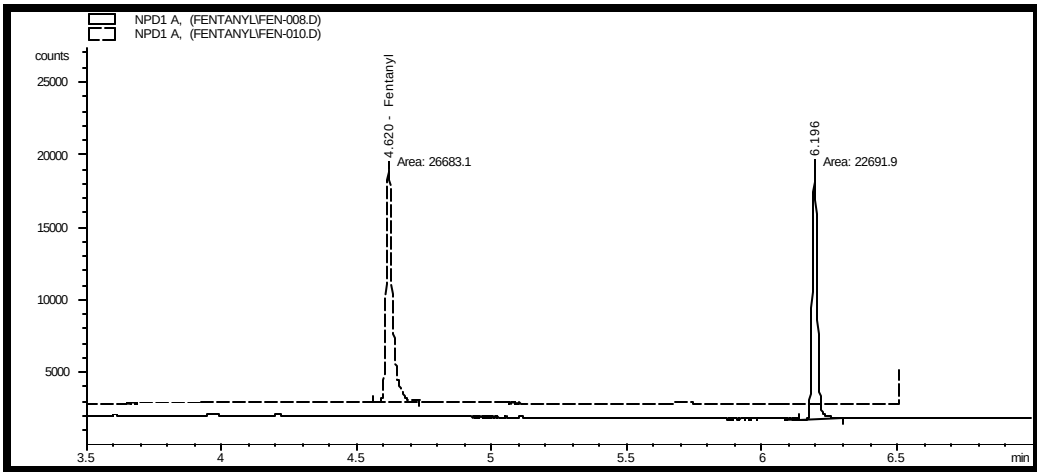


Figure -17: Overlaid chromatograms of fentanyl at initial oven temperature 140°C(t_R = 6.196 min.) and 200°C (t_R = 4.620 min.) using splitless injection mode.

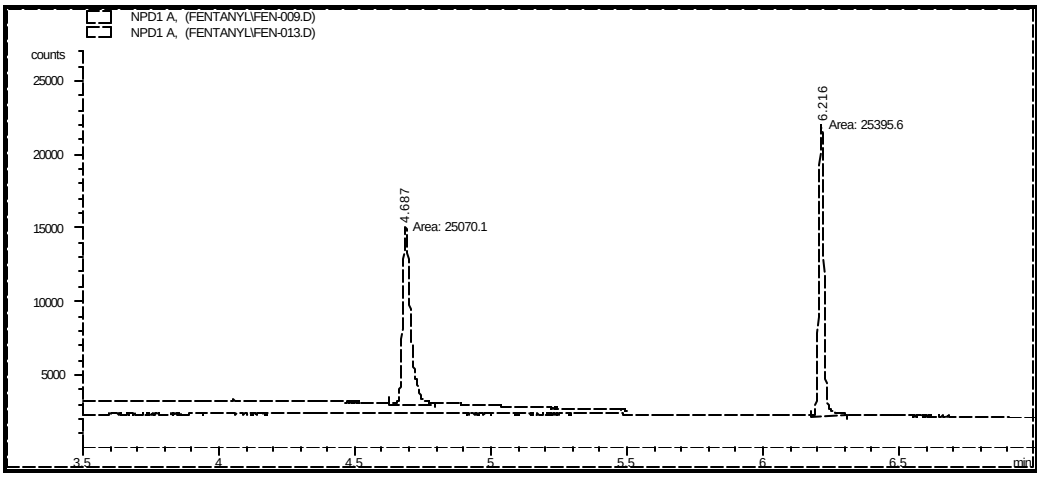


Figure -18: Overlaid chromatograms of fentanyl at initial oven temperature 140°C(t_R = 6.216 min.) and 200°C (t_R = 4.687 min.) using split injection mode.

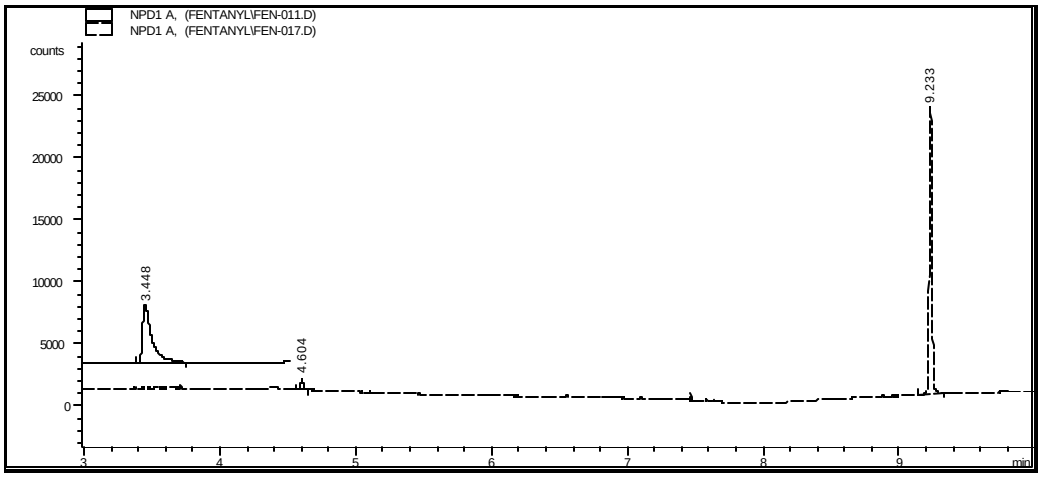


Figure -19: Overlaid chromatograms of fentanyl at initial oven temperature 80°C(t_R = 9.233 min.) and 240°C (t_R = 3.448 min.) using splitless injection mode.

Of the four oven temperature programs the program with initial oven temperature of 140°C and an oven temperature ramp rate of 40°C/min. was considered to be suitable for finalising the assay procedure as this program has an acceptable tur n-around time and the peak shapes of the analytes were very good.

The formal validation of the assay method was begun.

6 VALIDATION OF THE GC/NPD ASSAY METHOD

6.1 Validation of the GC/NPD assay procedure

6.1.1 System Performance Verification

Preparation of the System Performance Verification Sandard (SPVS)

System performance verification (SPV) samples were included at the beginning, middle and end of each batch of samples assayed to monitor and ensure reproducible performance of analytical system throughout its use during a particular study. It is used to indicate whether the instrument in use is working properly or not and to give a green light to proceed with the assaying of the next batch of samples.

The SPVS samples containing approximately 250 ng/ml each of fentanyl and sufentanil were prepared as follows:

Fentanyl SPVS solution A

- 100 µl fentanyl citrate injection solution (50 µg/ml fentanyl equivalent)
- add 100 µl of 10 M NaOH
- extract with 10 ml toluene by shaking vigorously for 5 minutes
- allow to settle
- transfer toluene layer to a scintillation vial

- dry the extract over about 1 g anhydrous Na_2SO_4
- store the SPVS A solution containing approximately 500 ng/ml fentanyl in a refrigerator

Sufentanil SPVS B solution

- 1 ml sufentanil citrate injection solution (5 µg/ml fentanyl equivalent)
- add 100 µl of 10 M NaOH
- extract with 10 ml toluene by shaking vigorously for 5 minutes
- allow to settle
- transfer toluene layer to a scintillation vial
- dry the extract over about 1 g anhydrous Na_2SO_4
- store the SPVS B solution containing approximately 500 ng/ml sufentanil in a refrigerator

To prepare the final SPVS solution, equal volumes of solutions A and B were combined to contain approximately 250 ng/ml of each component.

System Performance Verification

A batch of SPVS samples was run using three injection volumes (1, 2 and 3 µl) to assess the linearity of the autosampler injector. The following system performance verification run-sheet was prepared:

Table-10: System performance verification run-sheet

Vial #	Sample	Vol.(ul)	Vial #	Sample	Vol.(ul)	Vial #	Sample	Vol.(ul)	Vial #	Sample	Vol.(ul)
1	SPVS	3	9	SPVS	2	17	SPVS	1	25	SPVS	3
2	Toluene	3	10	Toluene	3	18	Toluene	3	26	Toluene	3
3	SPVS	2	11	SPVS	1	19	SPVS	3	27	SPVS	2
4	Toluene	3	12	Toluene	3	20	Toluene	3	28	Toluene	3
5	SPVS	1	13	SPVS	3	21	SPVS	2	29	SPVS	1
6	Toluene	3	14	Toluene	3	22	Toluene	3	30	Toluene	3
7	SPVS	3	15	SPVS	2	23	SPVS	1			
8	Toluene	3	16	Toluene	3	24	Toluene	3			

A summary of the system performance verification run is presented in Tables-11a&11b

Table-11a: Summary of SPV data

Replicate Number	Vol. Injected (ul)	Amount injected (pg)	Peak Areas			Response factor	
			Fentanyl	Sufentanil	Ratio	Fentanyl	Sufentanil
1	1	250	2847.73	2950.20	0.965	0.0878	0.085
	2	500	6634.14	6821.09	0.973	0.0754	0.073
	3	750	10286.60	10385.50	0.991	0.073	0.072
2	1	250	2311.28	2439.98	0.947	0.108	0.102
	2	500	4922.42	4894.62	1.006	0.102	0.102
	3	750	10138.30	10135.80	1.000	0.074	0.074
3	1	250	2332.42	2226.80	1.047	0.107	0.112
	2	500	5784.80	5689.88	1.017	0.086	0.088
	3	750	11443.90	11278.30	1.015	0.066	0.067
4	1	250	2263.93	2148.25	1.054	0.110	0.116
	2	500	5532.53	5500.99	1.006	0.090	0.091
	3	750	10943.80	10634.30	1.029	0.069	0.071
5	1	250	2129.52	2138.35	0.996	0.117	0.117
	2	500	7361.19	6275.58	1.173	0.068	0.080
	3	750	9791.17	9622.69	1.018	0.077	0.078

Table-11b: Summary of SPV data

Vol. Injected	1ml	2ml	3ml	1ml	2ml	3ml
Replicate No.	Fentanyl Peak Area			Sufentanil Peak Area		
1	2847.73	6634.14	10286.60	2950.20	6821.09	10385.50
2	2311.28	4922.42	10138.30	2439.98	4894.62	10135.80
3	2332.42	5784.80	11443.90	2226.80	5689.88	11278.30
4	2263.93	5532.53	10943.80	2148.25	5500.99	10634.30
5	2129.52	7361.19	9791.17	2138.35	6275.58	9622.69
MEAN	2376.98	6047.02	10520.75	2380.726	5836.43	10411.32
% CV	11.56	15.84	6.31	14.31	12.66	5.88

Vol. Injected	1ml	2ml	3ml	1ml	2ml	3ml
Replicate No.	Fentanyl Retention Time			Sufentanil Retention Time		
1	6.142	6.146	6.145	6.413	6.418	6.417
2	6.114	6.142	6.164	6.416	6.415	6.441
3	6.147	6.148	6.145	6.420	6.421	6.419
4	6.143	6.142	6.142	6.415	6.414	6.415
5	6.151	6.163	6.143	6.424	6.439	6.417
MEAN	6.139	6.148	6.147	6.417	6.421	6.421
% CV	0.24	0.14	0.15	0.068	0.16	0.17

The CV% of the peak area ratios for the 1,2 and 3 µl injections were 4.8, 7.0 and 1.5 % respectively. Thus, overall the 3 µl injection gave the most reproducible results.

A plot of the volume injected vs the peak area for fentanyl is presented in Figure-20.

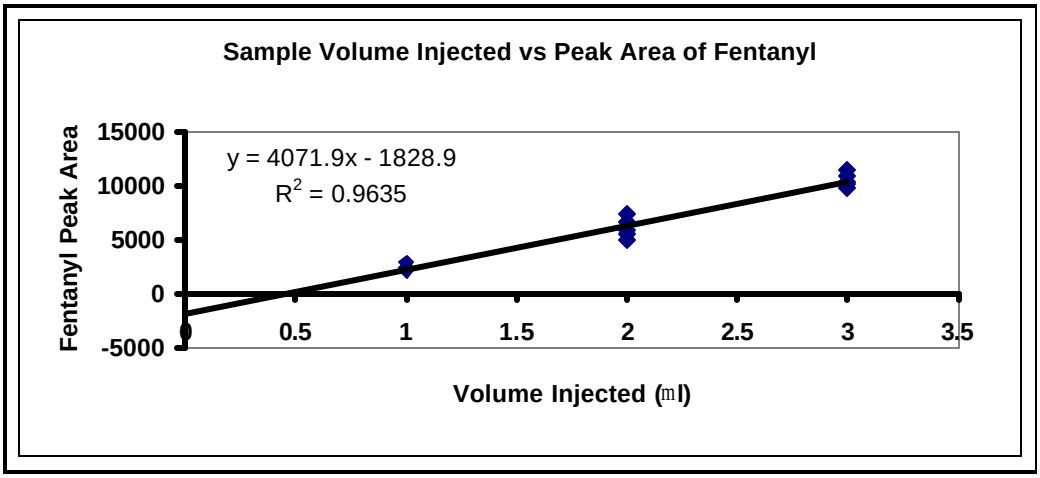


Figure -20: Linearity of autosampler injection

The performance verification data indicate that the instrument performance is acceptable but should actually be better. The somewhat poor performance of the autosampler injections is probably due to the fact that it is a relatively old instrument with a number of worn parts. The linearity data presented graphically indicates good injection linearity but the intercept on the volume axis indicates loss of sample equivalent to about 0.5 µl. It would be interesting to find out how and why that occurs.

The performance verification batch run was repeated with the initial oven temperature at 200°C and oven temperature ramp of 40°C/min. The results are presented in Tables-12a and -12b.

Table-12a: Summary of SPV data

Replicate Number	Vol. Injected (ul)	Amount injected (pg)	Peak Areas			Response factor	
			Fentanyl	Sufentanil	Ratio	Fentanyl	Sufentanil
1	1	250	3510.63	3572.55	0.983	0.071	0.070
	2	500	11092.20	10778.30	1.029	0.045	0.046
	3	750	18822.20	19018.70	0.990	0.040	0.039
2	1	250	3689.71	3590.46	1.028	0.068	0.070
	2	500	12833.20	12425.20	1.033	0.039	0.040
	3	750	21154.60	20204.50	1.047	0.035	0.037
3	1	250	3254.61	3148.62	1.034	0.077	0.079
	2	500	10977.50	10292.90	1.067	0.046	0.049
	3	750	19709.00	18796.30	1.049	0.038	0.040
4	1	250	3233.23	3233.28	1.000	0.077	0.077
	2	500	9997.71	9637.65	1.037	0.050	0.052
	3	750	17075.90	16456.60	1.038	0.044	0.046
5	1	250	3312.86	3300.47	1.004	0.075	0.076
	2	500	11022.50	10392.20	1.061	0.045	0.048
	3	750	17865.20	17165.90	1.041	0.042	0.044

Table-12b: Summary of SPV data

Vol. Injected	1ml	2ml	3ml	1ml	2ml	3ml
Replicate No.	Fentanyl Peak Area			Sufentanil Peak Area		
1	3510.63	11092.20	18822.20	3572.55	10778.30	19018.70
2	3689.71	12833.20	21154.60	3590.46	12425.20	20204.50
3	3254.61	10977.50	19709.00	3148.62	10292.90	18796.30
4	3233.23	9997.71	17075.90	3233.28	9637.65	16456.60
5	3312.86	11022.50	17865.20	3300.47	10392.20	17165.90
MEAN	3400.208	11184.62	18925.38	3369.076	10705.25	18328.4
CV %	5.75	9.17	8.41	5.98	9.77	8.22

Vol. Injected	1ml	2ml	3ml	1ml	2ml	3ml
Replicate No.	Fentanyl Retention Time			Sufentanil Retention Time		
1	4.511	4.526	4.614	4.783	4.800	4.892
2	4.509	4.449	4.499	4.781	4.771	4.773
3	4.506	4.509	4.508	4.777	4.783	4.775
4	4.502	4.497	4.496	4.772	4.771	4.796
5	4.510	4.497	4.494	4.782	4.770	4.767
MEAN	4.508	4.4966	4.522	4.779	4.779	4.8006
CV %	0.081	0.64	1.14	0.095	0.27	1.09

The CV % of the peak area ratios for the 1,2 and 3 µl injections were 2.4, 1.6 and 2.1 % respectively. Thus, all three injection volumes gave comparable results.

The results obtained with this oven temperature program are very similar to the first performance verification.

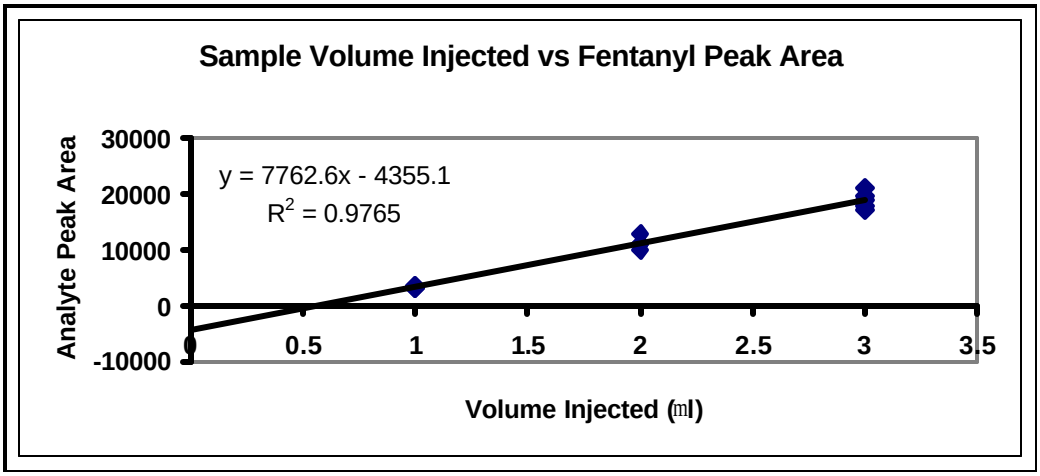


Figure -21: Linearity of autosampler injection

This concluded the system performance verification.

Calibration Line

The much shorter turn-around time achieved with the oven temperature program used in the latter method and the general good shape of the chromatograms was decisive in the choice of this method being the one to be validated. To confirm the reproducibility and extraction efficiency (recovery) of the method, extracts of fentanyl-plasma calibration standards in the concentration range from 0.19 to 100 ng/ml were extracted using the preferred extraction procedure and the reconstituted extracts injected onto the chromatograph column. The results are presented in Table-13, and the calibration curve in Figure-22.

Table -13: Fentanyl calibration standard data

Code	Conc. (ng/ml)	Fentanyl Peak Area	ISTD(50ng/ml) Peak Area	Peak Area Ratio	S/N
STD A	100	144695	53029	2.73	
STD B	50	73491.7	59403.6	1.24	
STD C	25	32741.1	52430.7	0.624	
STD D	12.5	17252.5	58389.1	0.295	
STD E	6.25	9722.51	65183.4	0.149	
STD F	3.125	3838.04	49384.3	0.078	
STD G	1.56	1907.37	54798	0.035	
STD H	0.78	954.55	52937.7	0.018	19
STD I	0.39	803.98	58047.5	0.014	11
STD J	0.19	406.65	59254.1	0.007	8

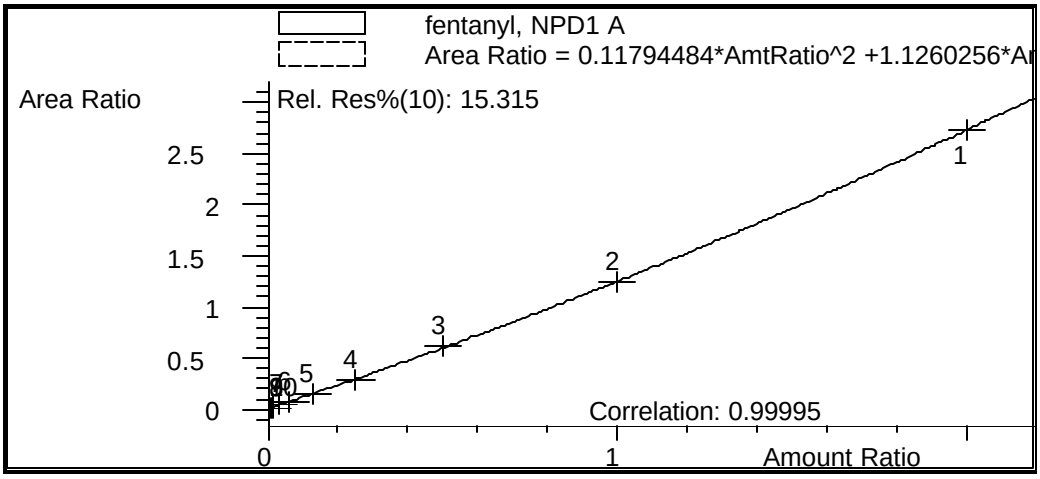


Figure -22: Calibration curve of fentanyl

Two representative chromatograms are presented in Figure-23.

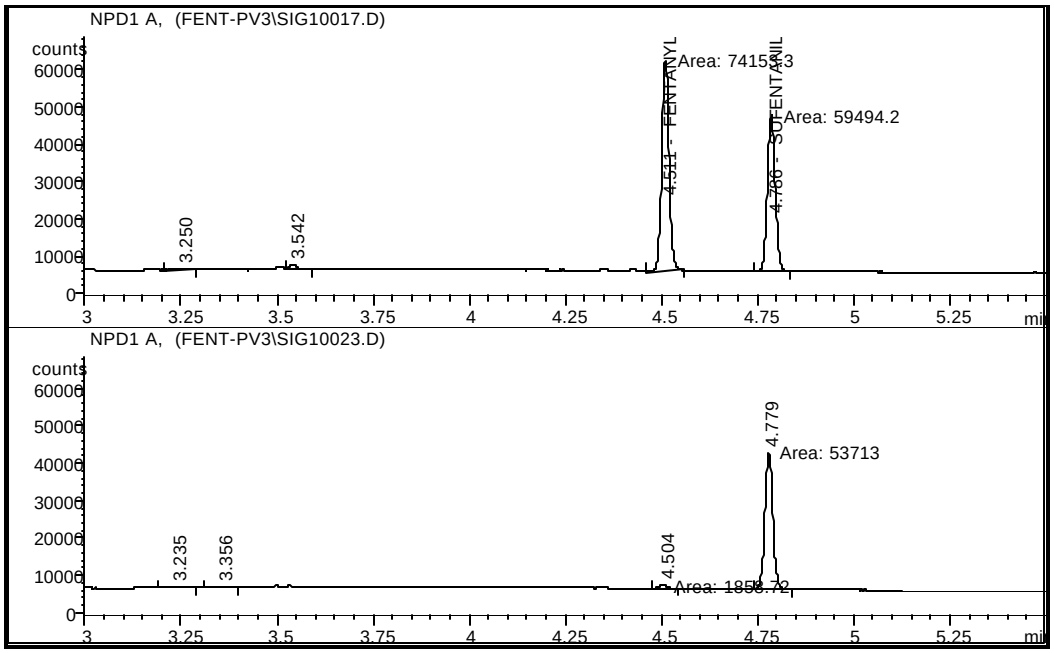


Figure -23: Chromatograms of 50 ng/ml & 0.78 ng/ml plasma-fentanyl extracts.

Reproducibility & recovery

Three plasma samples at high, medium, and low concentrations were extracted in triplicate, and injected into the GC-column. Results are presented in Table-14. Fentanyl solution in toluene with the expected concentration assuming 100 % extraction yield was also run to calculate recovery.

Table-14: Summary of reproducibility & recovery data.

	Fentanyl Peak Area		
Concentration / Replicates	**50 ng/ml**	**6.25 ng/ml**	**0.78 ng/ml**
1	42037.00	4515.99	846.86
2	51765.70	5454.10	825.04
3	52323.20	4985.04	751.35
MEAN	48708.63	4985.04	807.75
% CV	11.88	9.41	6.20
Fentanyl stock soln. peak area	57124.76	5747.40	628.79
% Recovery	85.27	86.74	119.50

The method is reproducible and the extraction efficiency is good, except at the lowest concentration where the recovery is a bit high, 120 %.

Having concluded the method pre-validation satisfactorily, the way was clear to proceed with the formal method validation.

6.1.2 Preparation of Plasma Calibration Standards

The calibration standards and quality control standards were prepared by two different analysts following a SOP of the laboratory. The author of this dissertation prepared the calibration standards.

According to the published literature Cmax was expected to be ~ 50 ng/ml, and previously published assay methods achieved a LLOQ ~ 0.2 ng/ml. It was therefore decided that the method should be validated from 2 x Cmax (100 ng/ml) down to the LLOQ (0.19 ng/ml).

Before proceeding with the preparation of the calibration standards and quality controls, the volume of plasma required had to be calculated. This was done on a calculation spreadsheet available in the laboratory.

Table-15: Calculated volume of plasma needed for preparation of STDs and QCs required in validation.

Phase	Sets of STDs (A)	Sets of QCs (B)	Levels STDs (C)	Levels QCs (D)	Replicate STDs (E)	Replicate QCs (F)	Sample volume (G) in ml	Volume (ml)
Intra-day	1	1	10	9	2	6	1.2	88.8
Inter-day-I	1	1	10	9	1	6	1.2	76.8
Inter-day-II	1	1	10	9	1	6	1.2	76.8
							Total	242.4

Volume of plasma = A x C x E x G +B x D x F x G

Calibration standards were prepared in human plasma. The stock solution (SA) required to spike the highest concentration calibration standard, was fentanyl citrate injection solution (Sublimaze®, 50 µg/ml fentanyl equivalent) contained in a 2 ml ampoule. A pool of normal plasma (STD K) was spiked with 100 µl of the stock solution, and was serially diluted (1:1) with normal plasma to obtain the desired concentrations (see Table-16).

Table -16: Preparation of plasma calibration standards

Calibration Standard	Source Solution	A	B	C	D (ng/ml)
STD K	**Stock SA**	72.812	122.826	N/A	102
STD J	**STD K**	70.936	95.922	120.218	50.5
STD I	**STD J**	72.907	97.942	122.920	25.2
STD H	**STD I**	70.990	95.976	120.990	12.6
STD G	**STD H**	72.130	97.126	122.137	6.31
STD F	**STD G**	72.373	97.376	122.400	3.16
STD E	**STD F**	71.966	96.967	121.987	1.58
STD D	**STD E**	72.616	97.693	121.613	0.773
STD C	**STD D**	70.887	95.904	120.922	0.386
STD B	**STD C**	72.253	97.248	122.250	0.193

KEY: A = Mass of empty container, **B** = Mass of container + normal plasma , **C** = Total mass of container + normal + spiked plasma, **D** = concentration of analyte (ng/ml), Specific Gravity = 1.0269 Kg/L for plasma.

STD J represents Cmax with a concentration of 50.5 ng/ml, and the LLOQ is represented by STD B (0.193 ng/ml). The calibration standards were aliquoted (1.2 ml) into polypropylene tubes and stored at ~ -20°C

6.1.3 Preparation of Plasma Quality Control Standards (QCs)

Quality control standards (QCs) were prepared by spiking a pool of normal plasma (QC I) with the stock solution SA (Sublimaze®, 50 µg/ml fentanyl equivalent) and serially diluting with blank plasma to obtain the desired concentration (see Table-17).

Table-17: Preparation of plasma quality control standards (QCs)

Quality Control	Source Solution	A	B	C	D (ng/ml)
QC I	**S A**	72.230	240.226	N/A	91.5
QC H	**QC I**	70.800	154.808	238.814	45.8
QC G	**QC H**	72.310	156.312	240.348	22.9
QC F	**QC G**	72.372	156.380	240.385	11.4
QC E	**QC F**	71.100	155.100	239.123	5.72
QC D	**QC E**	72.563	156.580	240.576	2.86
QC C	**QC D**	70.980	178.984	238.966	1.02
QC B	**QC C**	72.242	156.253	240.251	0.511
QC A	**QC B**	72.954	156.972	240.980	0.255

KEY: A = Mass of empty container, **B** = Mass of container + normal plasma , **C** = Total mass of container + normal + spiked plasma, **D** = concentration of analyte (ng/ml), Specific Gravity = 1.0269 Kg/L for plasma.

The QCs were aliquoted (1.2 ml) into polypropylene tubes and stored at ~ - 20°C together with the calibration standards.

6.1.4 Extraction Procedure

The plasma samples were thawed unassisted at room temperature, and were pipetted (1 ml) into 10 ml ampoules. 100 µl of 10 M NaOH, 100 µl of 0.5 µg/ml sufentanil injection solution in water (ISTD), and 5 ml ethyl ether were added and the sample vortex mixed for 2 min. After centrifugation at 2000 rpm and 4°C for 2 min., the aqueous phase was frozen in an alcohol freezing bath at –29°C. The organic phase was then decanted into another 10 ml ampoule containing 3 ml of 1 N H_2SO_4, vortex mixed for 2 min. followed by centrifugation for 2 min. at 2000 rpm and 4°C. The aqueous phase was frozen and the organic phase discarded. To the aqueous phase, 0.5 ml of 10 M NaOH and 5 ml ethyl ether were added, vortex mixed for 2 min., and centrifuged for 2 min. at 2000 rpm and 4°C. The aqueous phase was frozen in an alcohol freezing bath at –29°C, and the organic phase decanted into 5 ml glass test tubes, where it was evaporated and the residue reconstituted with 100 µl of toluene. The extracts were transferred into autosampler vials containing a 200 µl micro glass insert, and 3 µl injected into the GC-column.

6.1.5 Instrumental and Chromatographic Conditions

Chromatography was performed on a Hewlett Packard 5890 series II Gas Chromatograph equipped with an autosampler (HP 7673) and a Nitrogen/Phosphorus selective detector (NPD).

A Chrompac CP-sil 8CB fused silica capillary column (30 m x 0.32 mm i.d. and 0.25 µm film thickness of 5 % phenyl plus 95 % dimethyl polysiloxane) was used with high purity helium as carrier gas at a constant column head pressure of 25 psi.

The gas chromatograph temperature program was as follows: Initial temperature was 200°C, held for 1 min., then ramped by 40°C/min. to 300°C and held for 2 min. The injector and detector temperature were set at 280°C. The injector was operated in the split mode (split ratio 13:1) with a purge delay time of 1 min., followed by a purge flow of 2.4 ml/min. Gas flow rates were He (carrier gas) 3.81 ml/min., H_2 3.1 ml/min., N_2 44 ml/min., and air at 115 ml/min.

6.1.6 Intra-batch Accuracy and Precision

The method was validated by analyzing plasma quality control samples six times at nine different concentrations to determine the accuracy and precision of the method.

All results were calculated using the PhIRSt* (Phoenix International Life Sciences, Montreal, Canada) chromatographic data-reporting package. Peak areas are electronically read automatically from the report files generated by the Analyst version 1.3 software. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed ordered tables with performance statistics per batch and later summarised to give overall study statistics. This package has been validated in Canada by the manufacturer to FDA requirements (PhIRSt USERS MANUAL, Version 2.0). With the PhIRSt* data processing program a large number of regression equations can be fitted to the calibration data:

REGRESSION ALGORITHMS	
Linear	$y = mx + b$
Linear (1/c)	$y = mx + b$
Linear ($1/c^2$)	$y = mx + b$
Log – Log	$\ln (y) = m \ln (x) + c$
Wagner	$\ln (y) = a (\ln (x)^2) + b \ln (x) + c$
Quadratic	$y = a (x^2) + b (x) + c$
Quadratic (1/c)	$y = a (x^2) + b (x) + c$
Quadratic ($1/c^2$)	$y = a (x^2) + b (x) + c$

Calibration graphs were constructed using the Wagner regression of the analyte peak area ratio vs nominal drug concentration. Several regression types were tested and Wagner regression gave the best results.

The quality control values were calculated from the standard regression curve with eight different concentrations from 0.773 to 100 ng/ml. Ten calibration standards were prepared (Table-16), but in calculating the QC values only eight were used. The reason was that the lowest concentration calibration standards, i.e. 0.386 and 0.193 ng/ml were rejected since the % deviations of their back-calculated values were outside the acceptance range. Thus the LLOQ was raised to STD D (0.733 ng/ml) and the QCs B & A were rejected automatically.

Intra-batch accuracy and precision were assessed by the assay of all calibration standards (except STD B & C that were rejected) in duplicate to produce one calibration curve and six replicates of all the prepared QCS (except QC A & B that were rejected) in a single batch of assays. The intra-batch accuracy and precision of the assay procedure were assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas to get two different quantitation methods.

Accuracy is expressed as recovery of the analyte as % nominal while the precision is expressed as the CV %. For a valid method the intra-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nominal should be b/n 85 % - 115 %) over most of the range, and within 20 % of nominal concentration at the LLOQ. For a valid method the

intra-batch precision is required to be less than 15 % (i.e. CV % should be less than 15 %) over most of the range, and less than 20 % at the LLOQ.

The method performed well using both quantitation methods (peak height and peak area). The peak height quantitation method gave the best results and was used for the statistical analysis of the two inter-batch validations.

The results of the intra-batch validation are summarized in tables 18 & 19 for quantitation by peak height ratio, and in tables 20 & 21 for the quantitation by peak area ratio.

6.1.6.1 Quantitation by Peak Height Ratios

Table-18: Back calculated concentrations of fentanyl based on peak height ratios

STD Code	Nominal Concentration (ng/ml)	Back calculated Concentration (ng/ml)	% Dev
STD K	102.000	101.549	-0.4
STD K	102.000	101.182	-0.8
STD J	50.500	52.664	4.3
STD J	50.500	52.141	3.3
STD I	25.200	21.684	-14.0
STD I	25.200	25.792	2.3
STD H	12.600	12.563	-0.3
STD H	12.600	13.655	8.4
STD G	6.310	6.175	-2.1
STD G	6.310	6.292	-0.3
STD F	3.160	3.055	-3.3
STD F	3.160	3.360	6.3
STD E	1.580	1.585	0.3
STD E	1.580	1.554	-1.7
STD D	0.773	0.777	0.6
STD D	0.773	0.768	-0.7

Quantification Method = Peak height ratio, Regression Equation = Wagner (a = 0.00897, b = 0.9997, c = -3.7369, R^2 = 0.99904)

Table-19: Summary of intra-batch quality control results based on peak height ratios

Code Nominal Replicates	QC I 91.5 ng/ml	QC I (Dil.) 91.5 ng/ml	QC H 45.8ng/ml	QC G 22.9ng/ml	QC F 11.4 ng/ml	QC E 5.72ng/ml	QC D 2.86 ng/ml	QC C 1.02 ng/ml
1	91.651	86.476	48.205	24.247	11.881	5.629	3.075	N/a
2	95.144	86.166	47.480	22.867	11.589	6.137	3.230	1.169
3	94.605	101.036	50.508	28.339	12.725	6.594	3.401	1.212
4	101.864	85.570	50.302	23.594	11.698	6.038	3.112	0.974
5	84.800	91.350	48.081	22.950	12.307	5.415	3.719	0.857
6	97.052	98.136	52.160	28.504	12.982	6.501	3.323	1.037
MEAN	94.19	91.46	49.46	25.08	12.20	6.05	3.31	1.05
%Nom	102.9	100.0	108.0	109.5	107.0	105.8	115.7	102.92
CV%	6.1	7.3	3.7	10.5	4.7	7.7	7.1	13.77

6.1.6.2 Quantitation by Peak Area Ratios

Table-20: Back calculated concentrations of fentanyl based on peak area ratios

STD Code	Nominal Concentration (ng/ml)	Back calculated Concentration (ng/ml)	% Dev
STD K	102.000	103.296	1.3
STD K	102.000	100.304	-1.7
STD J	50.500	51.048	1.1
STD J	50.500	51.739	2.5
STD I	25.200	22.692	-10.0
STD I	25.200	25.233	0.1
STD H	12.600	12.979	3.0
STD H	12.600	13.649	8.3
STD G	6.310	6.326	0.2
STD G	6.310	6.216	-1.5
STD F	3.160	3.062	-3.1
STD F	3.160	3.188	0.9
STD E	1.580	1.552	-1.8
STD E	1.580	1.579	-0.1
STD D	0.773	0.842	9.0
STD D	0.773	0.721	-6.7

Quantification Method = Peak area ratio, Regression Equation = Wagner (a = 0.007757, b = 0.992785, c = -3.750656, R^2 = 0.999195)

Table-21: Summary of intra-batch quality control results based on peak area ratios

Code Nominal Replicates	QC I 91.5 ng/ml	QC I (dil.) 91.5 ng/ml	QC H 45.8ng/ml	QC G 22.9ng/ml	QC F 11.4 ng/ml	QC E 5.72ng/ml	QC D 2.86 ng/ml	QC C 1.02 ng/ml
1	102.55	103.40	48.84	23.16	11.48	5.67	2.94	N/A
2	95.14	86.20	48.66	23.13	11.41	6.04	3.17	1.09
3	96.07	98.36	50.62	27.14	12.80	6.78	3.37	1.20
4	99.96	82.01	48.81	24.00	11.86	6.01	3.11	0.98
5	83.46	85.19	48.06	22.48	11.89	5.69	3.85	0.90
6	98.23	99.79	50.32	28.04	12.91	6.54	3.30	1.00
MEAN	95.90	92.49	49.22	24.66	12.06	6.12	3.29	1.032
%Nom	104.8	101.1	107.5	107.7	105.8	107.0	115.0	101.4
CV%	6.9	9.8	2.1	9.5	5.4	7.4	9.5	11.10

Note: QC I (dil) means QC I was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results were adjusted by a dilution factor of 2 in order to obtain the correct nominal concentration.

6.1.7 Inter-batch Accuracy and Precision

Inter-batch accuracy and precision were assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards (from Cmax to LLOQ) and six replicates of each of the quality control standards (i.e. from highest, medium, and lowest concentrations) designated for use in the assay of samples of unknown concentrations. Five levels of QCs should be used. These are:

- Highest = 1.9 Cmax (QC H)
- High = 0.8 Cmax (QC G)
- Medium = 0.5 Cmax (QC E)
- Low = 2.3 x LLOQ (QC B)
- LLOQ =1.2 – 1.8 x STD B (QC A)

Due to the fact that QC B & A had to be discarded because of the high % deviation of the back-calculated calibration standards they were omitted in the assessment of accuracy and precision.

The inter-batch accuracy and precision of each of the batches were assessed separately by calculating the regression equation and constructing the calibration curve based on the best curve fitting equation as well as applying the criteria for inter-batch acceptance. The final inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 validations). Accuracy is expressed as the % difference between the nominal and calculated value or as % nominal of the analyte, while precision is expressed as the coefficient of variation (% CV). For a valid method the intra- and inter-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of the nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (i.e. % CV should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the first inter-batch validation with highest variation 7.6 % for QC E. But in the second inter-batch validation, though the % deviation for the calibration standards is within range, the % CV for QC G and QC E, 15.9 & 21.2 respectively, are too high.

The results are summarized in tables 22, 23, 24 and 25.

6.1.7.1 Inter-batch 1 Accuracy and Precision

Table-22: Back calculated concentrations of fentanyl based on peak height ratio

STD Code	Nominal Concentration (ng/ml)	Back calculated Concentration (ng/ml)	% Dev
STD J	50.500	50.183	-0.6
STD I	25.200	26.631	5.7
STD H	12.600	11.500	-8.7
STD G	6.310	6.546	3.7
STD F	3.160	3.103	-1.8
STD E	1.580	1.665	5.4
STD D	0.773	0.751	-2.8

Quantification Method = Peak height ratio, Regression Equation = Wagner (a = -0.002656, b = 1.03984, c = -3.694495, R^2 = 0.99877)

Note: STD C & B were omitted because the % deviations of their back-calculated concentrations were outside the acceptance criteria.

Table -23: Summary of quality control results for inter-batch 1 validation

Code Nominal Replicates	QC H 45.8 ng/ml	QC G (Dil.) 22.9 ng/ml	QC E 5.72ng/ml
1	48.512	23.722	5.615
2	51.741	23.814	5.412
3	46.427	23.491	5.531
4	49.653	23.118	5.727
5	50.317	22.261	6.263
6	54.236	24.404	6.495
MEAN	50.15	23.47	5.67
%Nom	109.5	102.5	99.2
CV%	5.4	3.1	7.6

6.1.7.2 Inter-batch 2 Accuracy and Precision

Table-24: Back calculated concentrations of fentanyl based on peak height ratio

STD Code	Nominal Concentration (ng/ml)	Back calculated Concentration (ng/ml)	% Dev
STD J	50.500	48.947	-3.1
STD I	25.200	25.400	0.8
STD H	12.600	13.203	4.8
STD G	6.310	6.743	6.9
STD F	3.160	2.904	-8.1
STD E	1.580	1.478	-6.5
STD D	0.773	0.822	6.4

Quantification Method = Peak height ratio, Regression Equation = Wagner (a = 0.004627, b = 1.002948, c = -3.5650025, R^2 = 0.99832)

Table-25: Summary of quality control results for inter-batch 2 validation

Code / Nominal / Replicates	QC H 45.8 ng/ml	QC G (Dil.) 22.9 ng/ml	QC E 5.72ng/ml
1	45.647	24.325	5.266
2	42.892	19.963	5.226
3	50.645	23.801	5.343
4	54.677	24.085	6.168
5	48.174	15.756	3.386
6	52.498	24.207	6.714
MEAN	49.09	22.02	5.35
%Nom	107.2	96.2	93.5
CV%	8.9	15.9	21.2

6.1.8 Summary of The Combined Quality Control Results for the 3 Validations

The combined quality control results are summarized in Table-26. The method performed well during the course of all three validations with the highest CV % of 13.34.

Table-26: Summary of the combined QC results for the 3validations

Validation Batch	Code Nominal / Replicates	QC H 45.8ng/ml	QC G 22.9ng/ml	QC E 5.72ng/ml
Intra-batch Validation	**1**	48.84	23.16	5.67
	2	48.66	23.13	6.04
	3	50.62	27.14	6.78
	4	48.81	24.00	6.01
	5	48.06	22.48	5.69
	6	50.32	28.04	6.54
Inter-batch 1 Validation	**1**	48.512	23.722	5.615
	2	51.741	23.814	5.412
	3	46.427	23.491	5.531
	4	49.653	23.118	5.727
	5	50.317	22.261	6.263
	6	54.236	24.404	6.495
Inter-batch 2 Validation	**1**	45.647	24.325	5.266
	2	42.892	19.963	5.226
	3	50.645	23.801	5.343
	4	54.677	24.085	6.168
	5	48.174	15.756	3.386
	6	52.498	24.207	6.714
	MEAN	49.56	23.52	5.75
	% nom	108.22	102.73	100.49
	CV %	6.04	11.59	13.34

6.1.9 Stability Assessment

6.1.9.1 Freeze-thaw Stability

This stability test is done to ensure that the samples remain stable after they were subjected to multiple freeze-thaw cycles during the process of the study. This can be done by thawing samples at high, medium and low concentrations unassisted and storing them frozen again for at least 12-24 hrs. The cycle were repeated twice and the samples are processed at the end of the third cycle and the results were compared with freshly prepared samples.

From the QCs prepared (Table-17) five replicates of each of 0.2 Cmax (i.e. QC F = 11.4 ng/ml), and Cmax (i.e. QC H = 45.8 ng.ml) were frozen at -20^{o}C and put through three freeze and thaw cycles (thawed unassisted at room temperature). The samples were then assayed together (thawed for the fourth time) during the intra-day validation batch. Results are summarized in Table-27.

Table-27: Freeze and thaw stability assayed at 11.4 & 45.8 ng/ml

Nominal Conc. (ng/ml)	Assayed Conc. (ng/ml)	% Nominal
11.4	11.65	102.19
11.4	11.49	100.79
11.4	11.18	98.07
11.4	11.56	101.40
11.4	9.46	82.89
MEAN	11.1	97.37
Std. Dev	0.9	8.04
CV %	8.3	-----
45.8	47.55	103.82
45.8	44.73	97.66
45.8	[58.06]	126.77
45.8	45.62	99.61
45.8	47.16	102.95
MEAN	49	106.17
Std. Dev	5.4	11.78
CV %	11.1	-----

By plotting the assayed concentrations (excluding the value in square brackets which is an outlier) against the nominal concentrations of the data in Table-27, a linear regression with

slope of 1.02 (see Figure- 24) and intercept of -0.6 was obtained, indicating that the effect of three freeze-thaw cycles on the measured fentanyl concentration is negligible.

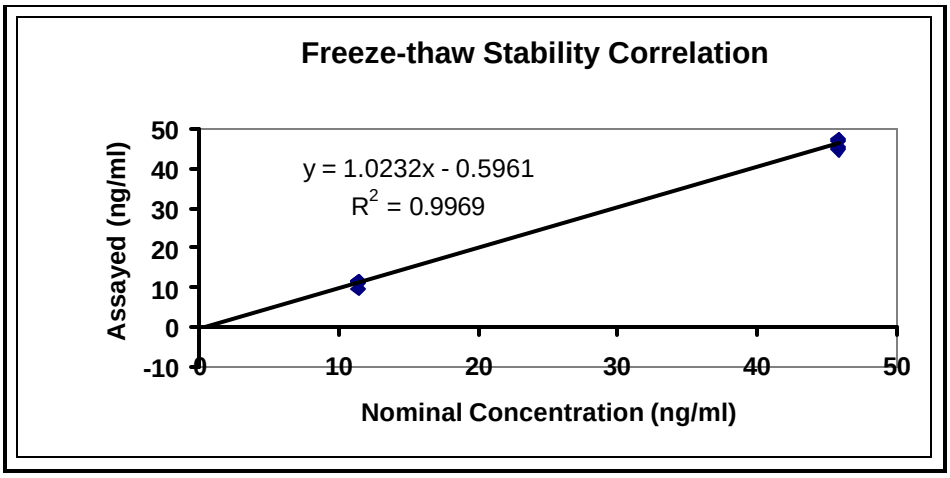


Figure -24: Freeze -thaw stability correlation

6.1.9.2 On-instrument Stability

The on-instrument stability test is done to ensure that the sample remains stable for the duration of time that it stays on the autosampler awaiting injection.

Eight stability samples of the same concentration (45.74 ng/ml) were extracted, and the extracts were pooled and aliquoted (1.2 ml). Problems with the detector bead were encountered during this validation run which caused large fluctuations in the detector sensitivity. Therefore, although the samples were injected in accordance with the intra-batch validation run-sheet, no conclusion could be drawn about the on-instrument stability on the basis of the results obtained. Unfortunately these assays could not be repeated at a later stage but the general impression gained during the method development and validation was that fentanyl and sufentanil are both very stable in toluene at room temperature and are therefore probably also stable in the reconstituted extracts on the autosampler awaiting injection. For the sake of completeness of the data generated during the intra-day validation, the results obtained with the on-instrument stability sample are nevertheless presented in Table-28 and in a graphical representation in Figure-25.

Table -28: Stability data of eight STAB samples injected at different time intervals

Replicates	Injection time	Time Difference	Cumulative Time (hr)	Analyte Peak Area	IS Peak Area	Peak Area Ratio
1	20:54		0.00	98560.00	76460	1.289
2	23:14	02:20:16	2.34	39260.00	29570	1.328
3	17:56	18:41:55	21.04	12960.00	11150	1.162
4	20:30	02:34:35	23.61	24470.00	20400	1.200
5	23:06	02:35:50	26.21	29890.00	29330	1.019
6	01:46	02:39:33	28.87	43180.00	37060	1.165
7	04:13	02:27:46	31.33	56380.00	43470	1.297
8	04:22	00:08:38	31.48	58300.00	43580	1.338
			Mean	45375.00	36377.50	1.22
			Std Dev	26421.20	19628.58	0.11
			CV %	58.23%	53.96%	8.90%

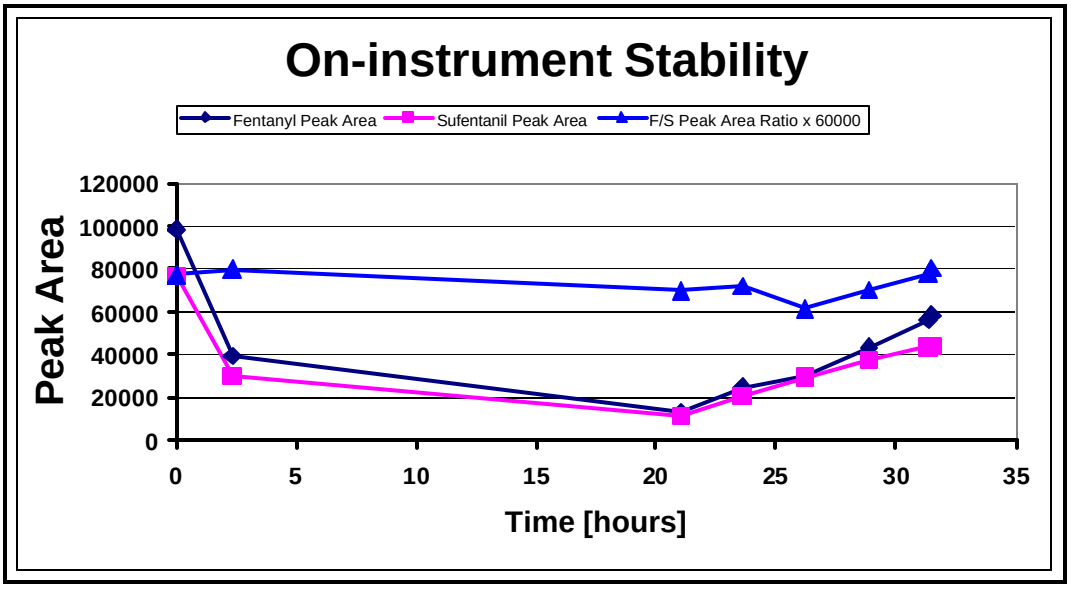


Figure -25: On-instrument stability graph.

Although the peak area of fentanyl and the sufentanil fluctuated, their ratio was relatively constant showing a CV = 8.9 %.

When the NP detector bead signal was monitored over a period of 30 SPVS injection runs its stability was found to be outside specification. Thus, the signal was distinctly erratic and at some stages even jumped to or fell abruptly to another level altogether.

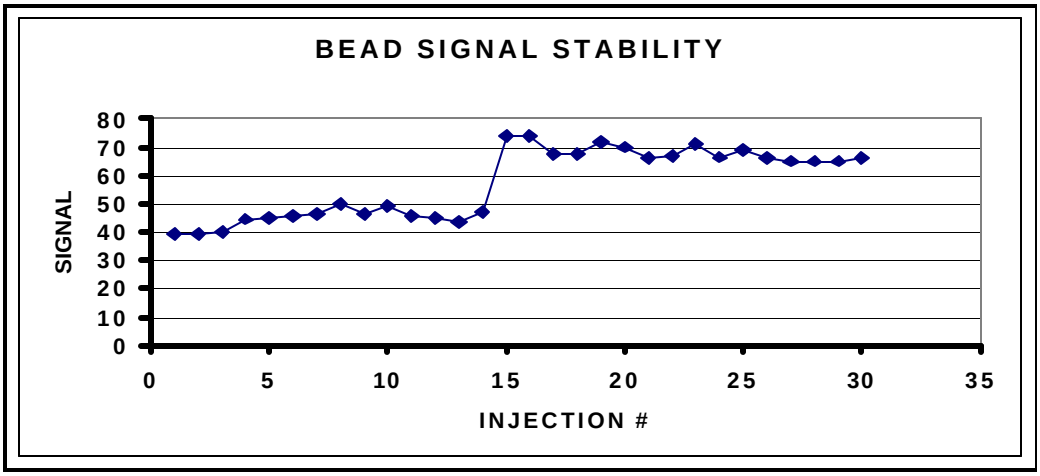


Figure -26: Bead signal stability graph

6.1.9.3 Bench-top Stability:

This test assesses the short-term stability of the analyte in a biological matrix at room temperature.

From the QCs prepared (Table-17) five replicates of each of 0.2 Cmax (i.e. QC F = 11.4 ng/ml) and Cmax (i.e. QC H = 45.8 ng/ml) were completely thawed unassisted at room temperature, and allowed to stand at room temperature overnight. These samples were then assayed with the inter-day-2 validation batch and the calculated concentrations were compared to the nominal concentrations. The results are summarized in Table-29.

Table-29: Bench-top stability measured in 11.4 & 45.8ng/ml plasma samples

High Concentration		Low Concentration	
Nominal (ng/ml)	**Measured (ng/ml)**	**Nominal (ng/ml)**	**Measured (ng/ml)**
45.8	46.55	11.4	9.88
45.8	44.14	11.4	11.24
45.8	49.81	11.4	11.36
45.8	48.46	11.4	11.89
45.8	47.17	11.4	9.40
Mean	47.23	**Mean**	10.75
Std Dev	2.13	**Std Dev**	1.06
%Nom.	103.1	**%Nom.**	94.3
CV%	4.51	**CV%**	9.85

By plotting the assayed concentrations against the nominal concentrations of the data in Ttable-29, a linear regression with slope of 1.06 (see Figure- 27) and intercept of -1.32 was obtained, indicating that the bench-top stability of fentanyl in plasma is good.

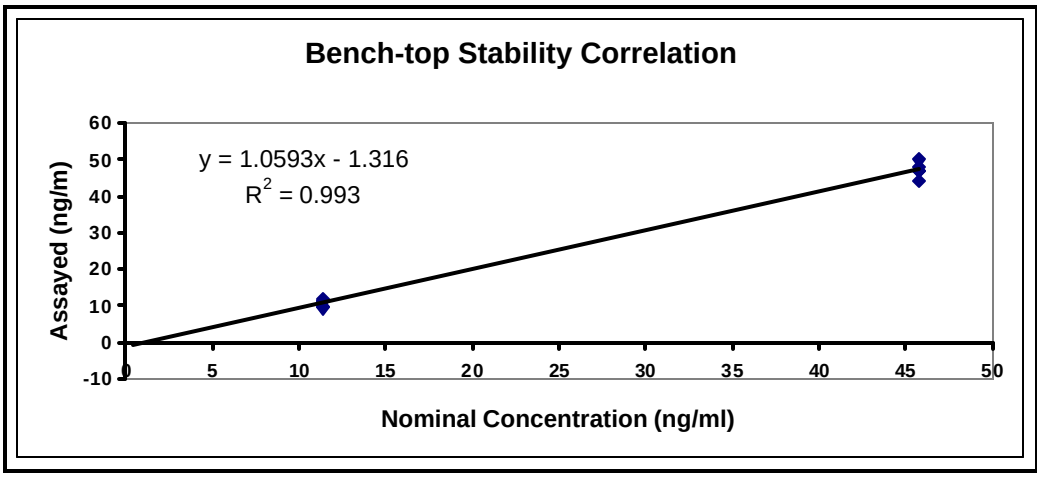


Figure -27: Bench-top stability correlation.

6.1.10 Specificity

No interfering or late eluting peaks were found in the six blank plasma chromatograms obtained from six different sources of plasma. Figure-28 is an example of a chromatogram of a blank plasma extract.

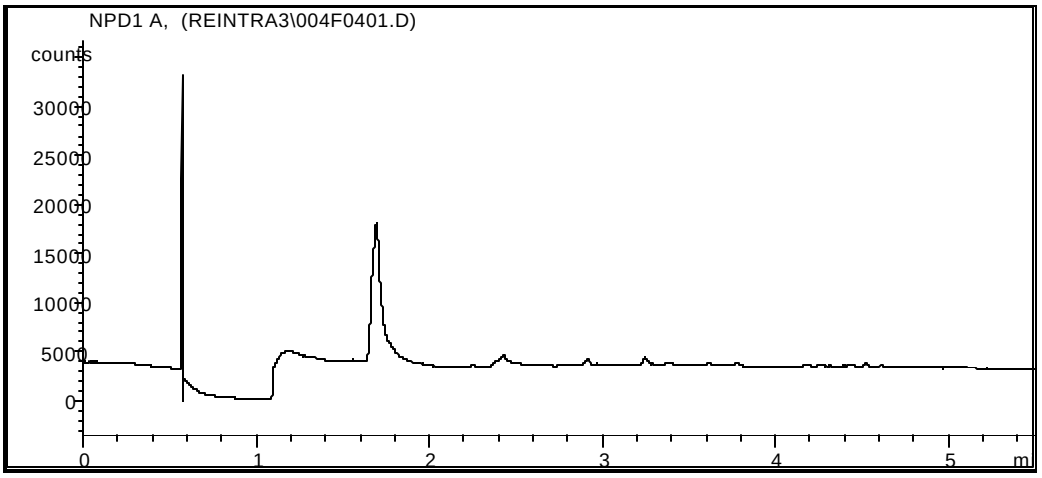


Figure -28: Chromatogram of a blank plasma extract

6.1.11 Sensitivity

The LLOQ, defined as that concentration of fentanyl which can still be determined with acceptable precision (CV % < 20) and accuracy (bias < 20 %), was found to be 0.773 ng/ml with a signal–to–noise ratio of ~12 (Figure-29a & b).

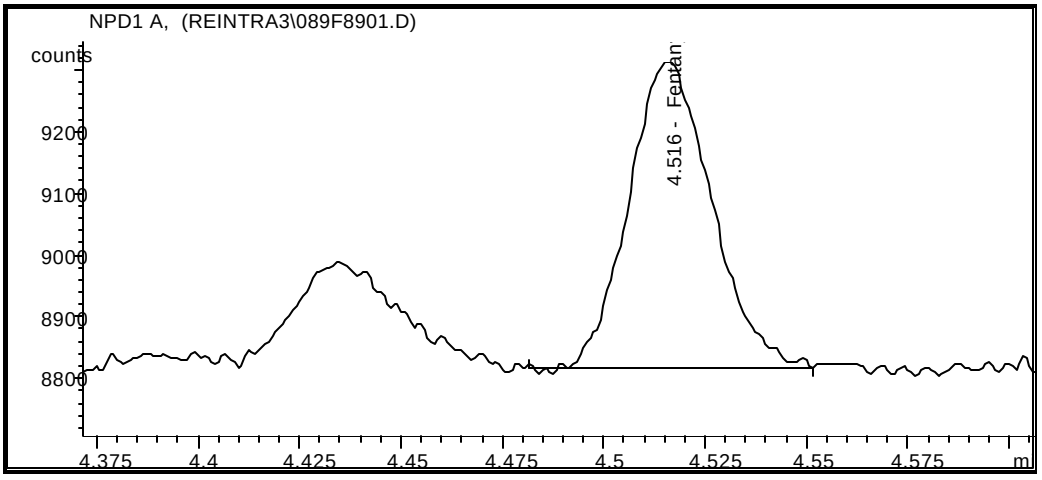


Figure - 29a: Chromatogram of an extract at the LLOQ, S/N ~12

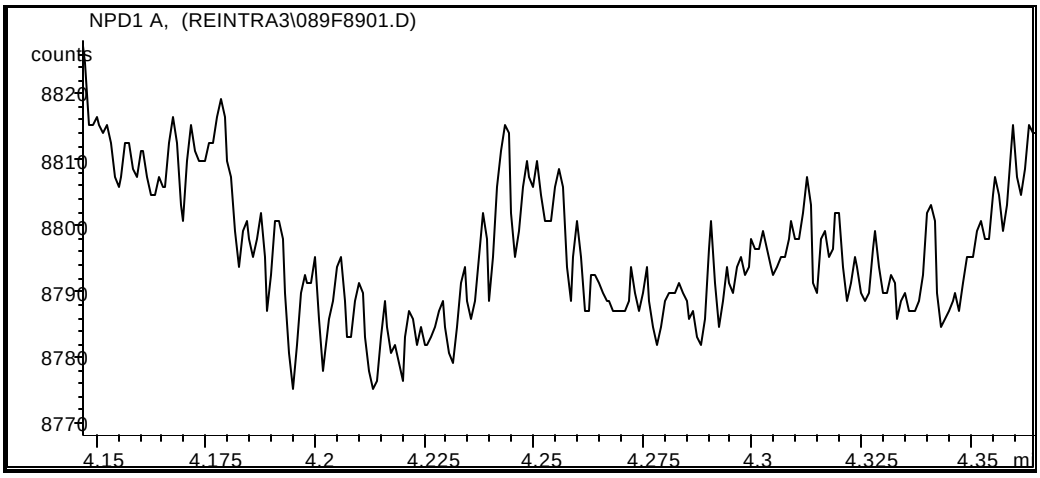


Figure -29b: Expansion of base-line noise

6.1.12 Recovery

Absolute recovery of a bioanalytical method is the measured response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of the analyte that is present in the sample (Bressolle, 1996).

$$\text{Absolute recovery} = \left(\frac{response\ of\ spiked\ plasma\ (processed)}{response\ of\ standard\ solution\ (unprocessed)} \right) \text{x } 100$$

Peak areas of three different quality control concentrations and theoretical peak areas obtained from the SPVS are used in calculating the recovery of the analyte according to the above-mentioned formula. Absolute recoveries of the analyte were determined in triplicate at high, medium, and low concentrations of the analytes in plasma and are summarized in Table-30.

Table-30: Absolute recovery of fentanyl using response factor areas

Sample	Mean of Peak Areas		Absolute Recovery(%)	CV(%)
	After Extraction	SPVS Values		
RCmax	66692.50	52987.55	125.86	4.66
RCave	31485.00	26493.77	118.84	3.99
RCmin	7451.50	6617.66	112.60	6.16

The recovery is high since there was concentration of analyte due to solvent evaporation over five days during which the extracts were standing on-instrument while the instrument was out of order.

Representative chromatograms of the plasma standards and QCs at high, medium and lower fentanyl concentration, and of a blank plasma are presented below.

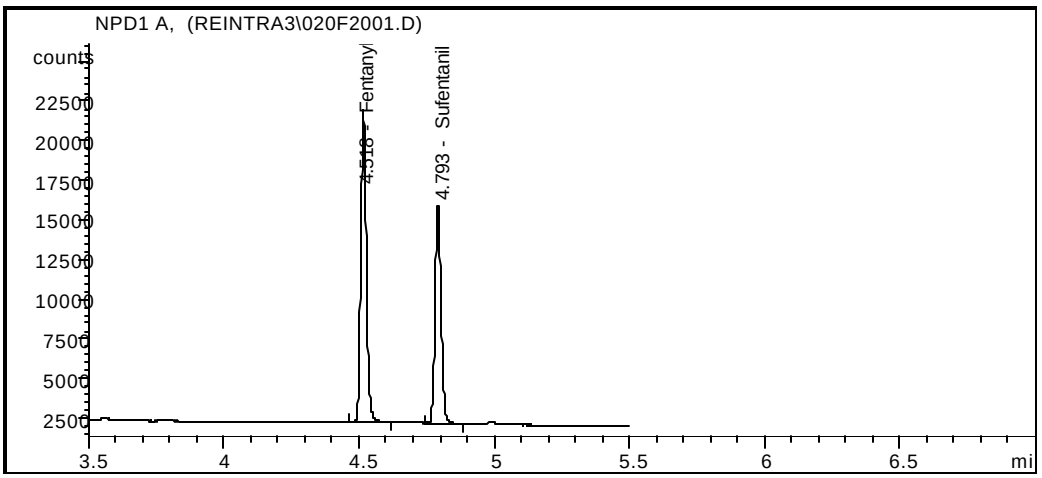


Figure -30: Chromatogram of an extract of STD J (50.5 ng/ml)

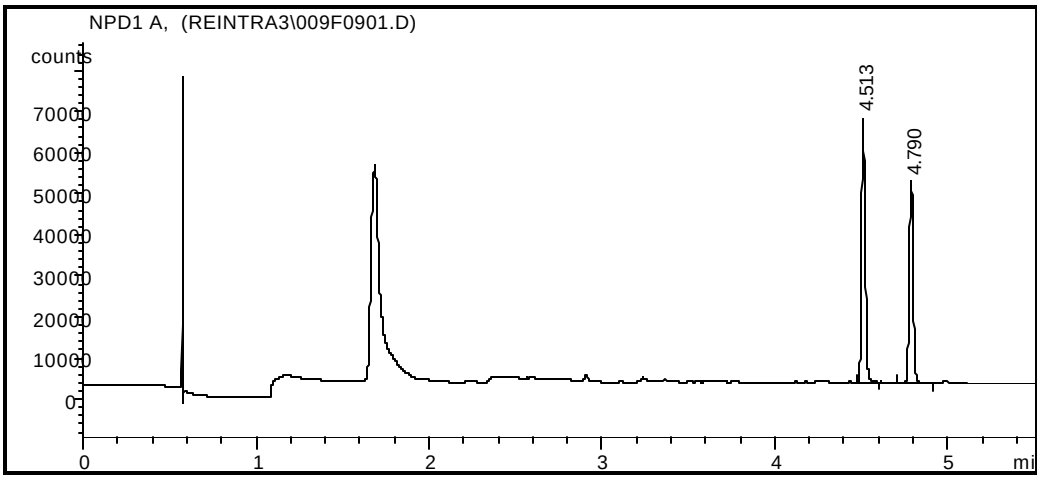


Figure -31: Chromatogram of an extract of QC H (45.8 ng/ml)

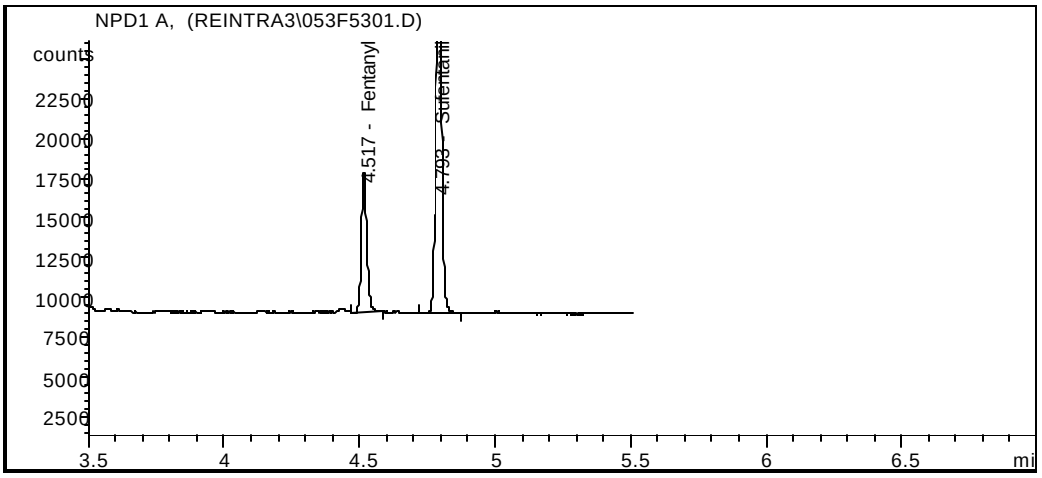


Figure -32: Chromatogram of an extract of STD H (12.6 ng/ml)

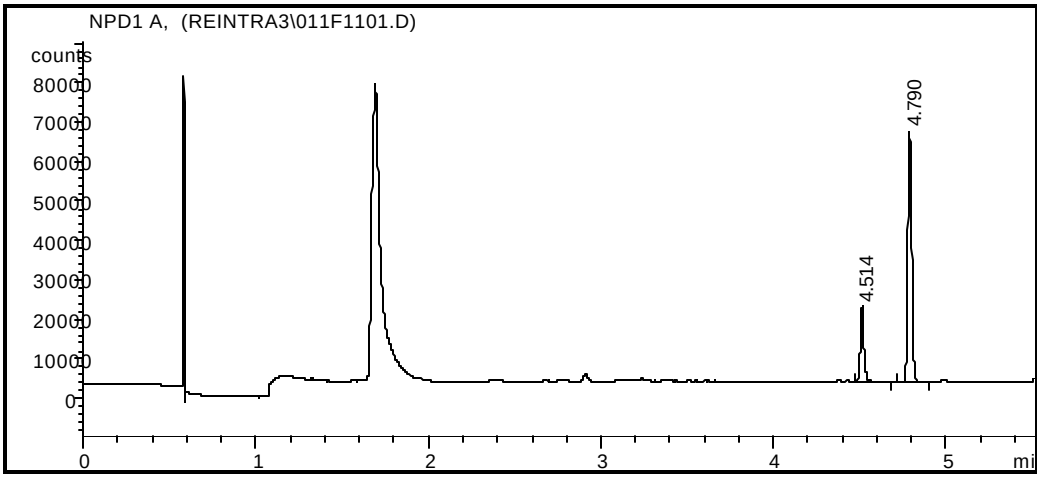


Figure -33: Chromatogram of an extract of QC F (11.4 ng/ml)

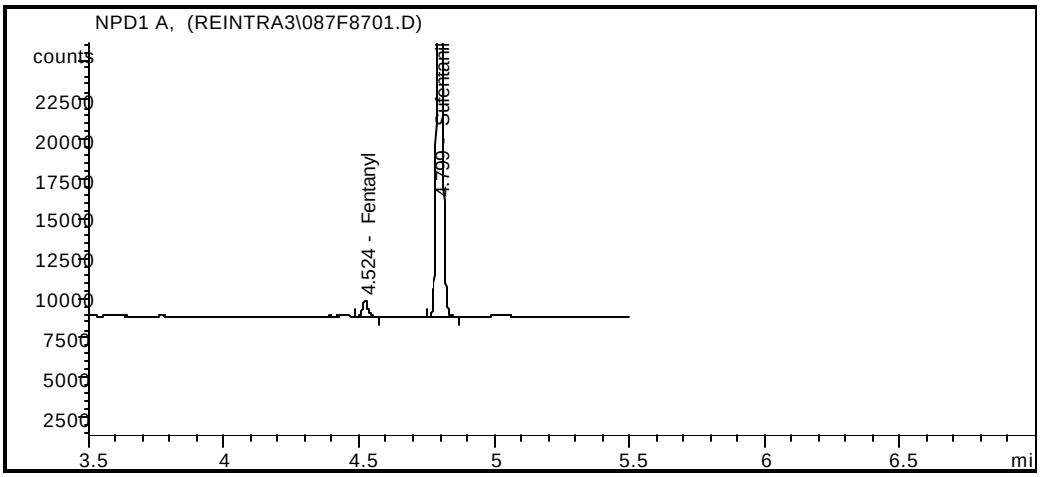


Figure -34: Chromatogram of an extract of STD E (1.58 ng/ml)

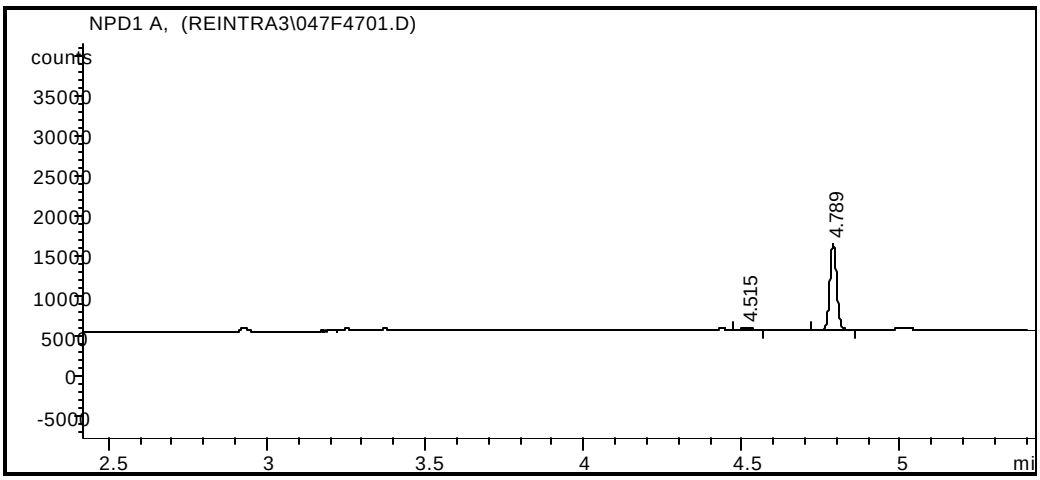


Figure -35: Chromatogram of an extract of QC C (1.02 ng/ml)

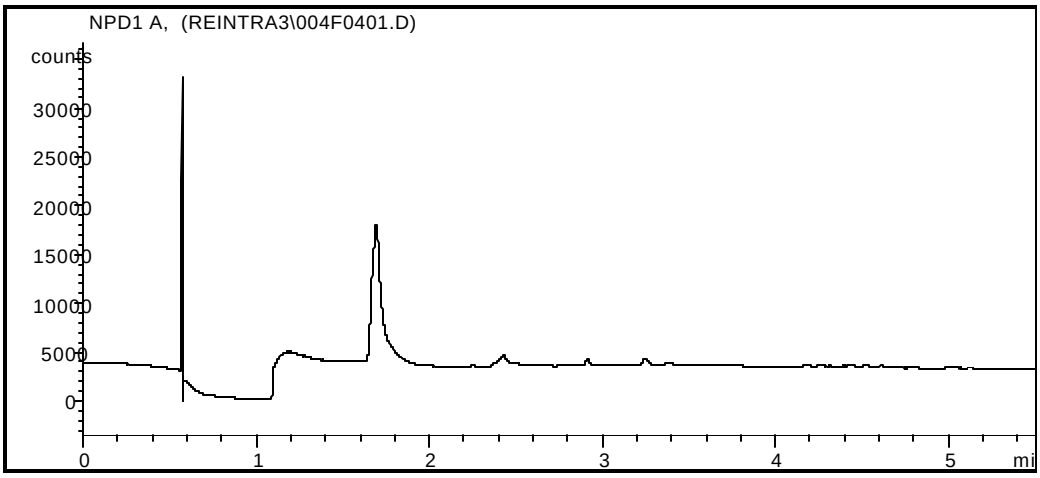


Figure -36: Chromatogram of an extract of blank plasma

6.1.13 Conclusion

The assay method for quantitative determination of fentanyl in human plasma was developed and validated using a gas chromatograph equipped with Nitrogen/Phosphorus Detector (NPD). At the method development stage, the LLOQ was estimated to be 0.19 ng/ml with S/N of 8, and the mean recovery (n = 3) at high, medium, and lower concentrations was ~ 85 %. But during the validation process of the method: the LLOQ was raised to 0.773 ng/ml

since the standards at lower concentrations (STD C =0.386 ng/ml & STD B = 0.193 ng/ml) were outliers and rejected.

During validation the mean recovery (n = 6) was ~ 120 % with CV < 6 %.

The reason for the high % deviation at lower concentration (specially STD C, STD B, QC B, and QC A), and for the high mean recovery could be due to:

- The malfunctioning of the syringe plunger, which resulted in aborting the sequence midway several times. As a result the extracts were forced to pass through five freeze-thaw cycles while storing them in a freezer and loading on instrument. In other words they also stayed on-instrument for five days.
- The instability of the bead signal, i.e. it was fluctuating too much that it affected the sensitivity of the method (peak areas and peak heights were fluctuating too).
- As the extracts stayed on-instrument for five days, while the auto-sampler was not thermostated there was solvent evaporation resulting in an increase of analyte concentrations. Therefore redilution of some of the extracts was done.

Still, with all these anomalies, the method performed satisfactorily.

Attention was given to the main problems stated in most of the literature, such as loss of analyte due to adsorption to glassware and extraction efficiency of organic solvents.

In this method no loss of analyte due to adsorption was observed probably because of the inertness of the disposable glass ampoules that were used during the extraction process.

The extraction efficiency of ethyl ether, n-butyl chloride, and toluene were comparable. Ethyl ether was chosen as the extracting solvent because of its low boiling point allowing evaporation of the solvent at a low temperature. Evaporation at temperatures higher than 50^{o}C reportedly causes adsorption of drugs to glassware.

The short turn-around time of 8.5 minutes makes this a good assay method for assaying large numbers of samples

7 LC-MS/MS ASSAY METHOD DEVELOPMENT

7.1 Preliminary Method Development by HPLC

To find a suitable mobile phase composition and optimize its pH for the method validation using LC/MS/MS system, a preliminary assay method development was performed by HPLC.

7.1.1 Instruments

HPLC analysis was performed using an Agilent 1100 Series quaternary pump combined with a Hewlett Packard (HP) 1100 series photodiode array detector (Germany), an HP 1100 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). The column used was Discovery® C_{18} bonded 5 µ silica, (15 cm x 2.1 mm) (Supelco, USA) with a mobile phase flow rate of 0.3 ml/min. An HP 1100 series thermostated column compartment (Germany) was used to control the column temperature.

Prior to any HPLC runs the absorbance of fentanyl citrate injection solution (50 µg/ml free base) was determined using a UV-spectrophotometer (see Table -31, and Figure-37).

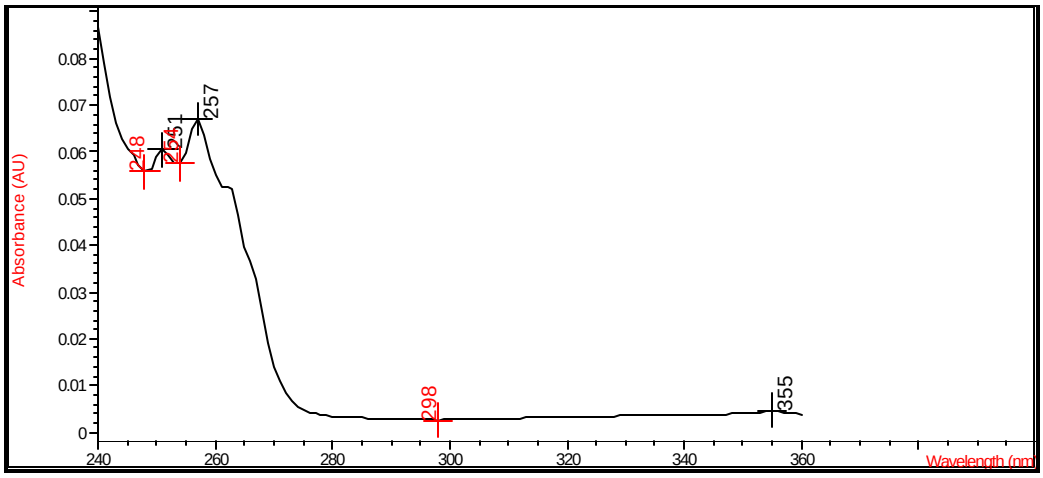


Figure -37: UV-spectrum of fentanyl

Table-31: Fentanyl absorbance maxima and minima

Name	Peaks (nm)	Abs(Au)	Valleys(nm)	Abs(Au)
Fentanyl	257.00	6.70E-02	248.00	5.60E-02
	251.00	6.10E-02	254.00	5.80E-02
	355.00	1.40E-02	298.00	1.30E-02

7.1.2 Mobile Phase Preparation

Two types of mobile phases were prepared.

Phosphate buffer based mobile phase

0.05 M H_3PO_4 was prepared by diluting 13.48 ml of concentrated H_3PO_4 (Assay =85 %; ρ = 1.71 kg/L) in water to a volume of 4 L. To prepare a pH 7 phosphate buffer, 0.05 M H_3PO_4 was titrated with 10 M NaOH to adjust its pH to 7.

Acetate buffer based mobile phase

0.05 M Acetic acid (AcOH) was prepared by diluting 11.35 ml of glacial acetic acid (Assay = 100 %; ρ =1.06 kg/L) in water to a volume of 4 L. To prepare a pH 7 acetate buffer, of 0.05 M AcOH was titrated with 25 % ammonia solution to adjust its pH to 7.

0.05 M H_3PO_4 and the pH 7 phosphate buffer were mixed in different proportions, their pH measured at room temperature, and % H_3PO_4 vs pH plotted (see Table-32 and Figure-38).

Similarly mixtures of 0.05 M AcOH and pH 7 acetate buffer were prepared, and the % of AcOH vs pH plotted (see Table -33, and Figure-39).

These graphs were used to determine the pH values of the buffer in mobile phases at different % compositions of acid and pH 7 buffer when mixed by the quaternary pump.

Table -32: pH of mixtures of 0.05 M H_3PO_4 & pH 7 phosphate buffer

Volume of 0.05M H3PO4 (ml)	Volume of pH 7 Buffer (ml)	% H3PO4	pH
0	10	0	7.00
1	9	10	6.79
2	8	20	6.45
3	7	30	6.06
4	6	40	4.45
5	5	50	2.93
6	4	60	2.60
7	3	70	2.42
8	2	80	2.35
9	1	90	2.16
10	0	100	2.11

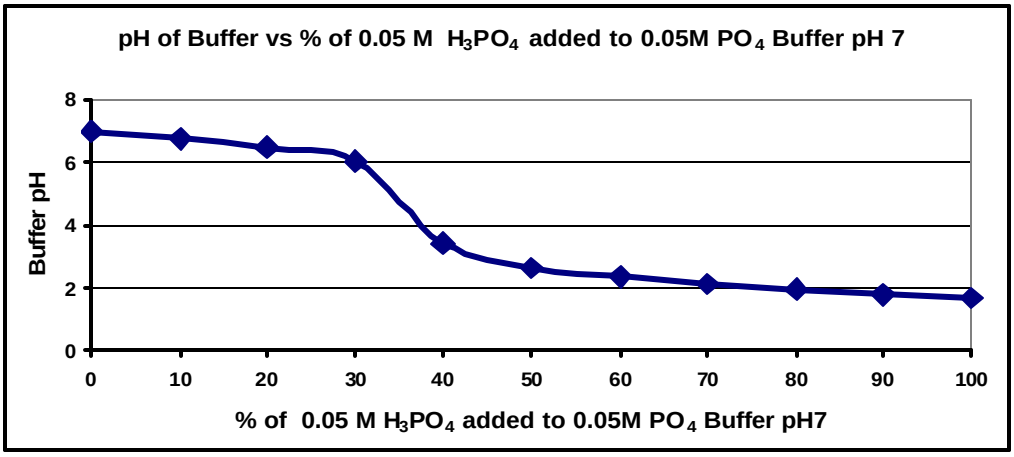


Figure -38: Graphical representation of the data in Table-32.

Table-33: pH of mixtures of 0.05 M AcOH & pH 7 acetate buffer

Volume of 0.05M AcOH (ml)	Volume of pH 7 Buffer (ml)	% AcOH	pH
0	10	0	7.00
1	9	10	5.70
2	8	20	5.30
3	7	30	5.00
4	6	40	4.90
5	5	50	4.70
6	4	60	4.50
7	3	70	4.30
8	2	80	4.10
9	1	90	3.80
10	0	100	3.20

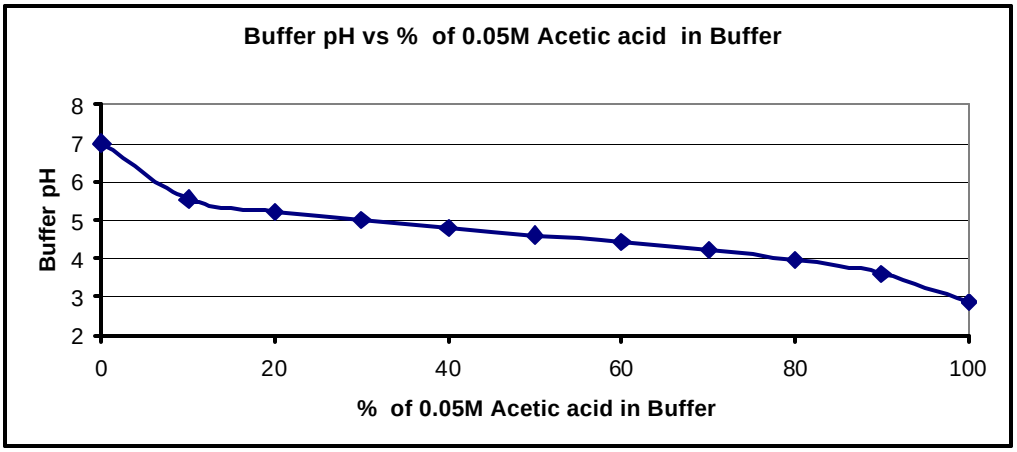


Figure -39: Graphical representation of the data in Table-33

Fentanyl Stock Solution Preparation

1.24 mg of fentanyl citrate salt (a white powder) was dissolved in 1 ml of deionised water to obtain a 1.24 mg/ml solution.

7.1.3 Chromatography

HPLC runs were performed at 20°C with UV-Vis detection at 257 nm. The volume injected was 5 µl of the 1.24 mg/ml fentanyl stock solution at a mobile phase flow rate of 0.3 ml/min.

Several gradient elution runs (10 % to 90 % in 20 min.) with methanol (MeOH) & phosphate buffer pH 7; as well as acetonitrile & phosphate buffer pH 7, were performed.

With MeOH & phosphate buffer peaks eluted at 20.047 & 23.464 min. (see Figure-40), and with acetonitrile & phosphate buffer at 21.021 & 22.405 min. (see Figure-41).

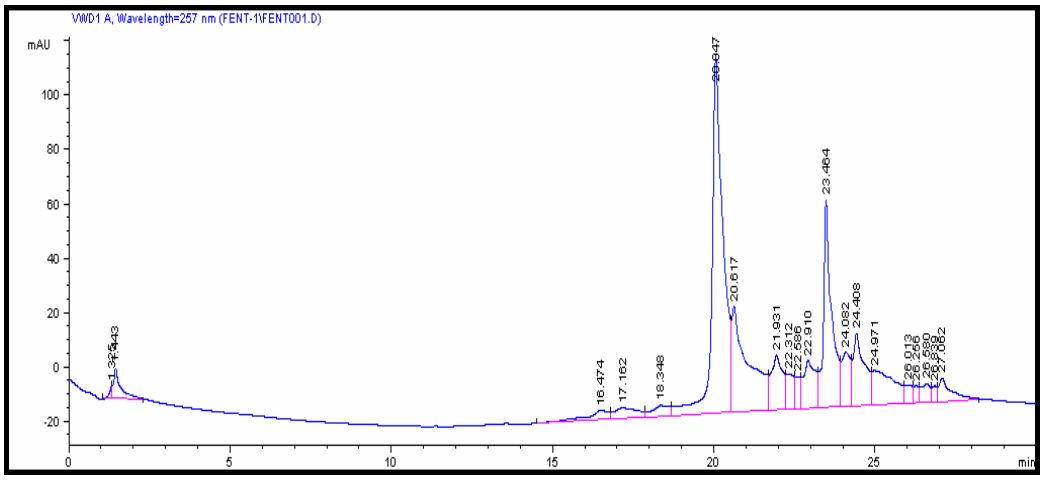


Figure -40: Gradient elution chromatogram of fentanyl with MeOH & phosphate buffer (pH 7).

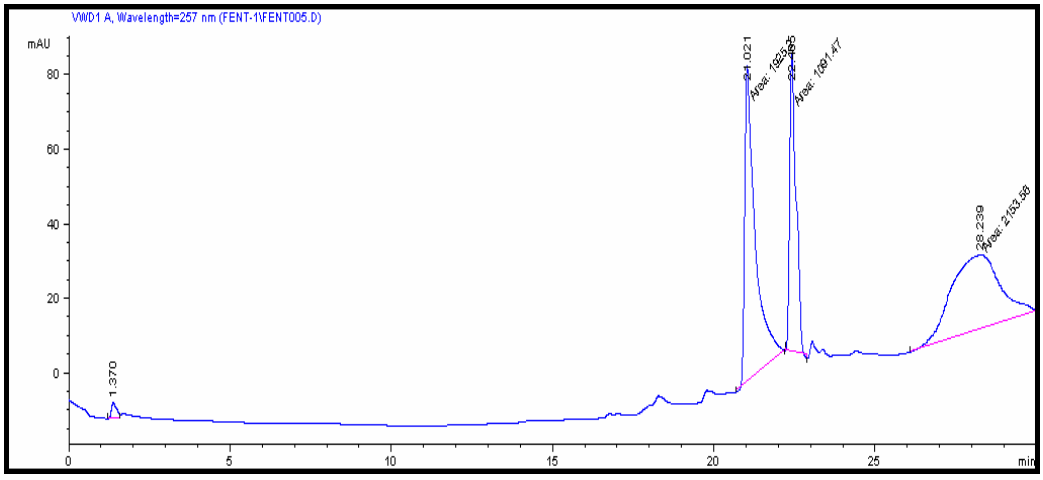


Figure -41: Gradient elution chromatogram of fentanyl with acetonitrile & phosphate buffer (pH 7).

In both chromatograms the peaks were obtained at approximately 70 % organic component. Isocratic elution with 70 % MeOH and 70 % acetonitrile respectively & phosphate buffer (pH 7) resulted in the following chromatograms.

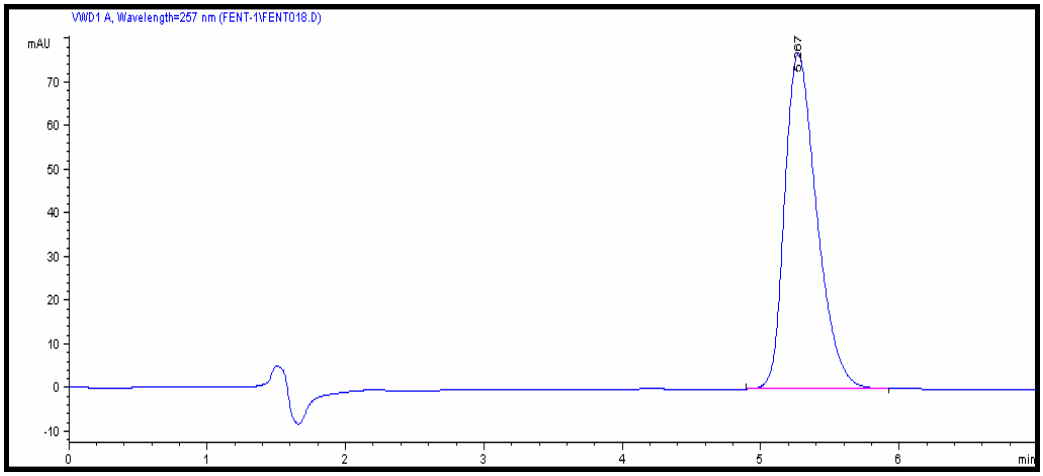


Figure -42: Isocratic elution chromatogram of fentanyl with 70 % MeOH in phosphate buffer (pH 7)

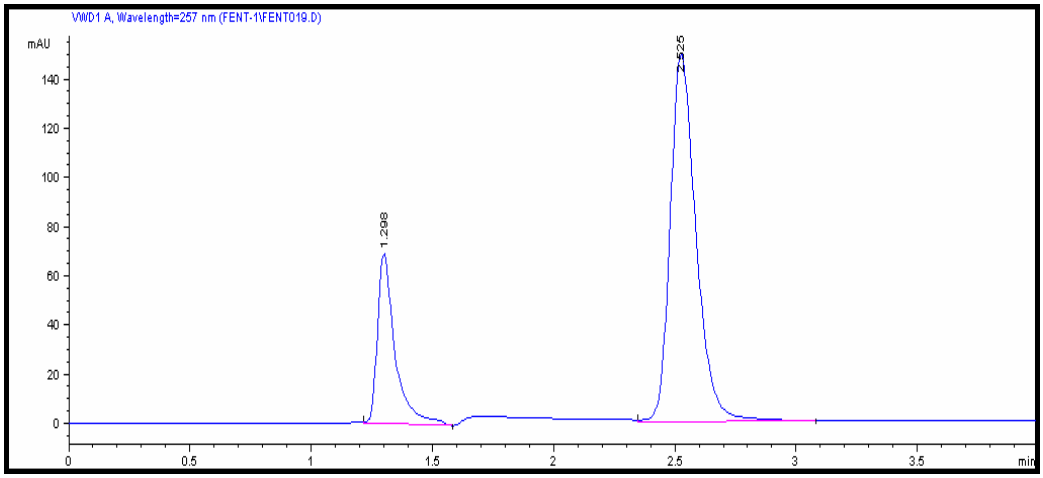


Figure -43: Isocratic elution chromatogram of fentanyl with 70 % acetonitrile in phosphate buffer (pH 7)

Since the main objective of these HPLC runs was to find a suitable mobile phase for the LC/MS/MS system where phosphate buffer or phosphoric acid can not be used, they were replaced with acetate buffer and acetic acid respectively. Several isocratic elution chromatograms using different mobile phase compositions formed with the quaternary HPLC pump were performed. A few representative chromatograms are given below.

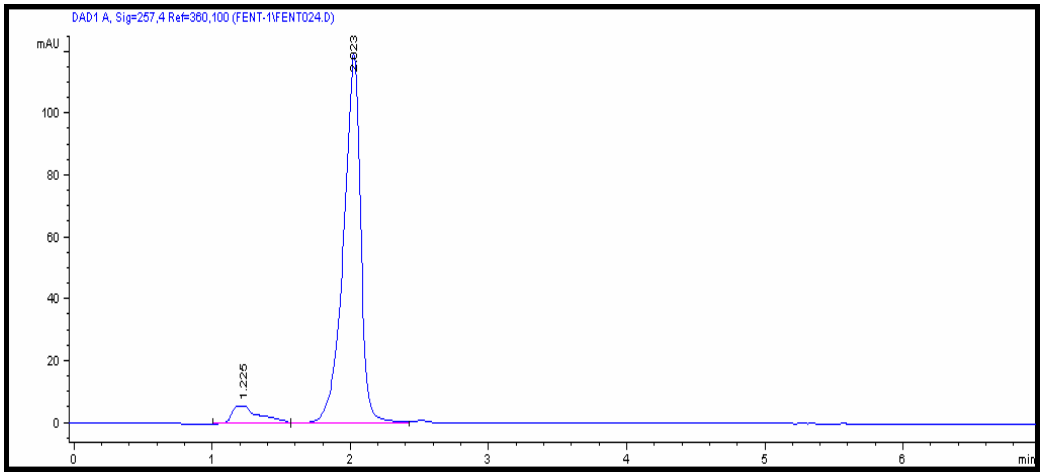


Figure -44: Chromatogram of fentanyl (isocratic elution with 70 % acetonitrile in acetate buffer pH 7).

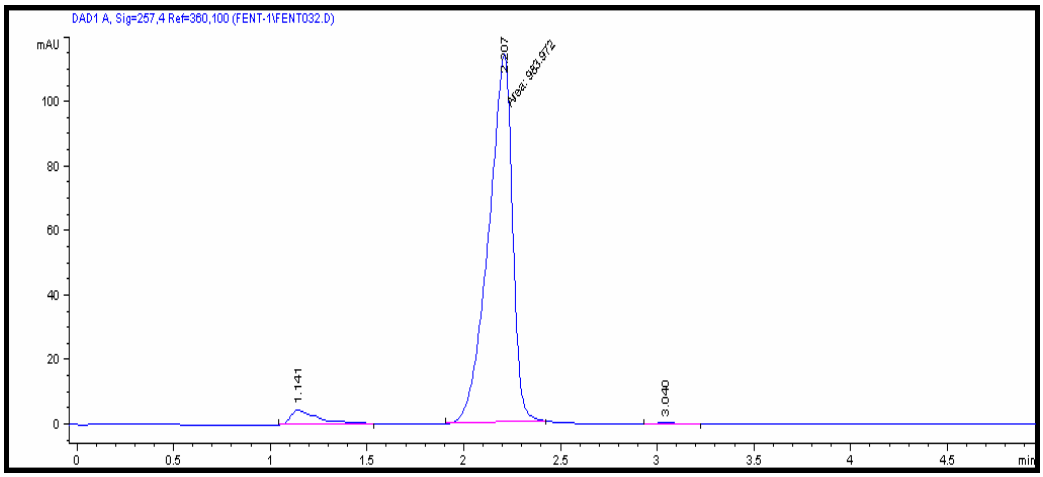


Figure -45: Chromatogram of fentanyl (isocratic elution with 65 % acetonitrile in acetate buffer pH 7)

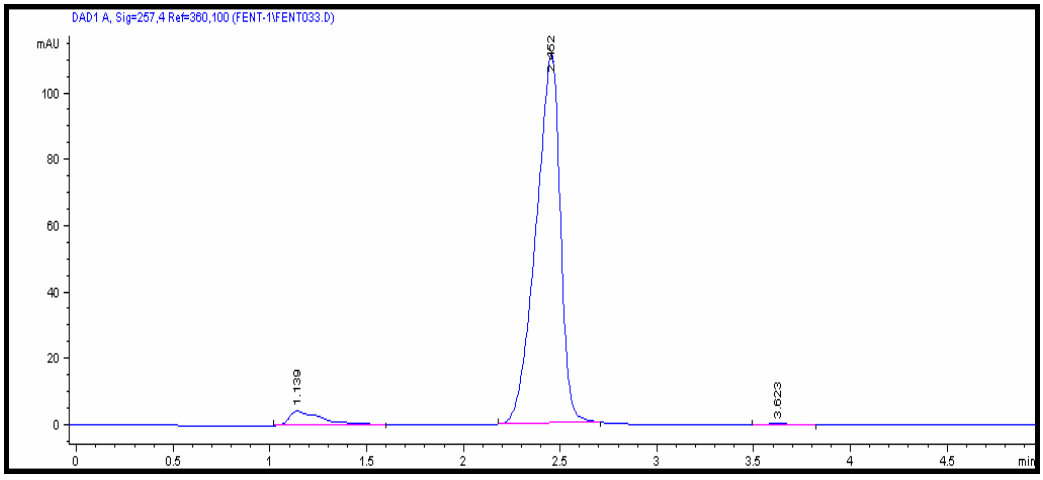


Figure -46: Chromatogram of fentanyl (isocratic elution with 60 % acetonitrile in acetate buffer pH 7.

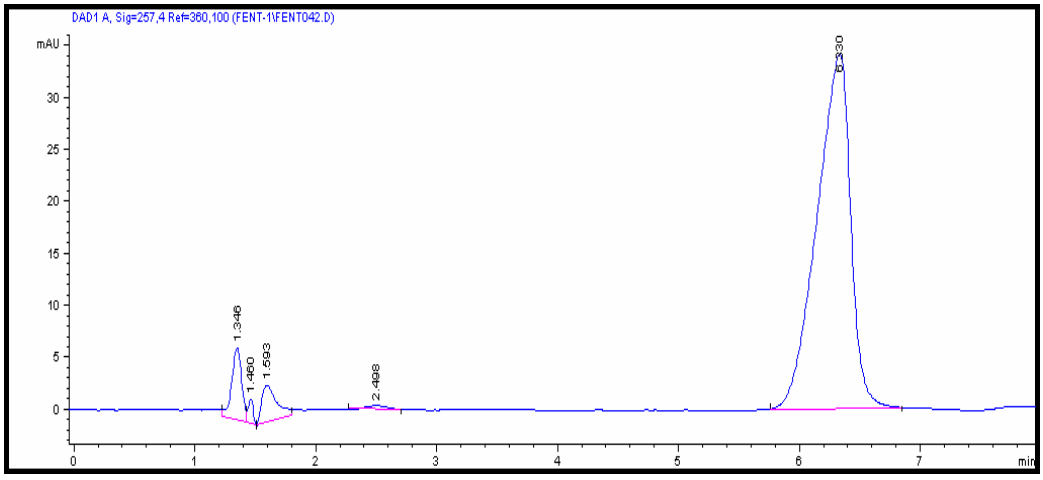


Figure -47: Chromatogram of fentanyl (isocratic elution with 60 % MeOH in acetate buffer p H 7.

Keeping acetonitrile and methanol constant at 60 %, acetate buffer based mobile phases at different apparent pH values were prepared as described above and chromatograms obtained of fentanyl. The effect of buffer pH on retention time are presented in the following tables and figures.

Table -34: Effect of buffer pH on t_R

60% Acetonitrile		60% Methanol	
pH	Rt	pH	Rt
		5.7	3.44
		5.3	2.65
5	1.68	5.0	2.34
4.7	1.6	4.7	2.09
4.3	1.46	4.3	1.95
4.1	1.43	4.1	1.88
3.8	1.39	3.8	1.78
3.2	1.38	3.2	1.57

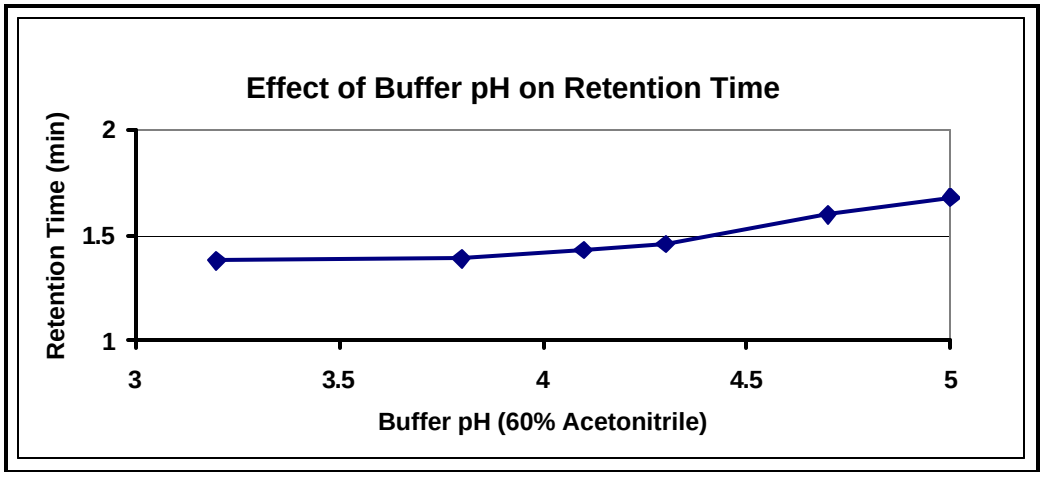


Figure -48: Graphical representation of the effect of buffer pH on t_R

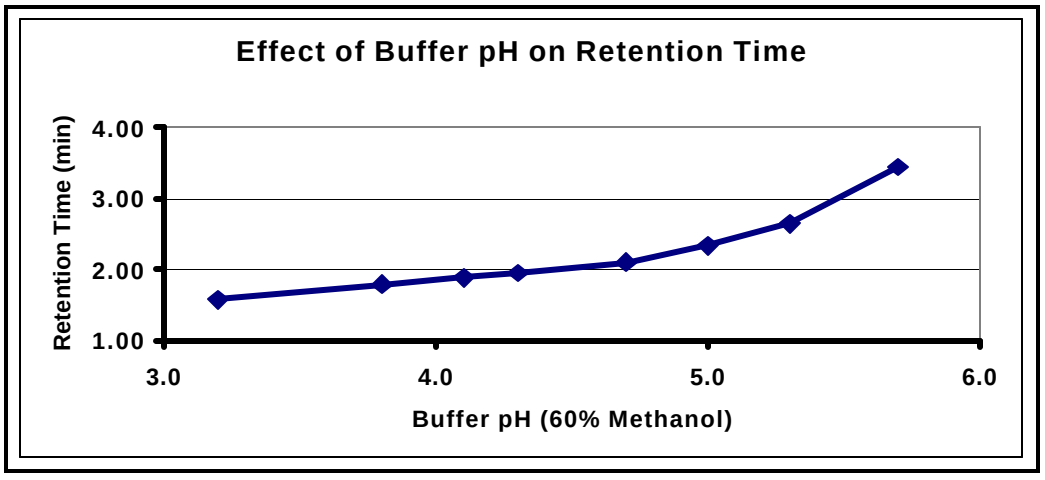


Figure -49: Graphical representation of the effect of buffer pH on t_R

TurboIonSpray ionisation is more efficient the higher the concentration of the organic modifier. Also, since fentanyl is a basic compound and ionises more efficiently at low than at high pH the mobile phase with methanol as organic modifier was chosen, because fentanyl has a higher retention time at low pH and high organic modifier concentration in the methanol based mobile phase than in the acetonitrile one.

In the procedures used during the experiments explained above the mobile phase composition was determined by the relevant low pressure solvent switching valves of the quaternary pump. Due to uncertainties inherent in the delivery of the relevant solutions by the switching valves, as well as possible incomplete mixing, especially at low delivery volumes, the above results could only be considered as preliminary indicators of the optimal values of the various mobile phase parameters which affect the quality of the chromatograms. Taking into account the results obtained, it was decided to use methanol as organic modifier at a level of 60 % v/v of the mobile phase and to prepare mobile phases for further optimisation as follows:

- To a volume of organic modifier (say 600 ml) was added 400 ml 0.05 M acetic acid.
- The resultant solution's pH was then adjusted drop wise with 25 % ammonia solution while stirring vigorously on a magnetic stirrer and monitoring the pH with a pH meter. In this manner a mobile phase with the required apparent pH could be produced very reproducibly.

An isocratic mobile phase (60 % MeOH & 40 % of 0.05M AcOH) was prepared by mixing 600 ml MeOH with 400 ml of 0.05 M AcOH, and transferred into four 250 ml bottles. Their pH was then adjusted with 25 % liquid ammonia to 4.0, 4.5, 5.0, and 6.7, respectively. Chromatographic runs were performed using the four mobile phases, and the best results were obtained at pH 4.5 and 6.7 (see Figure-50 & 51).

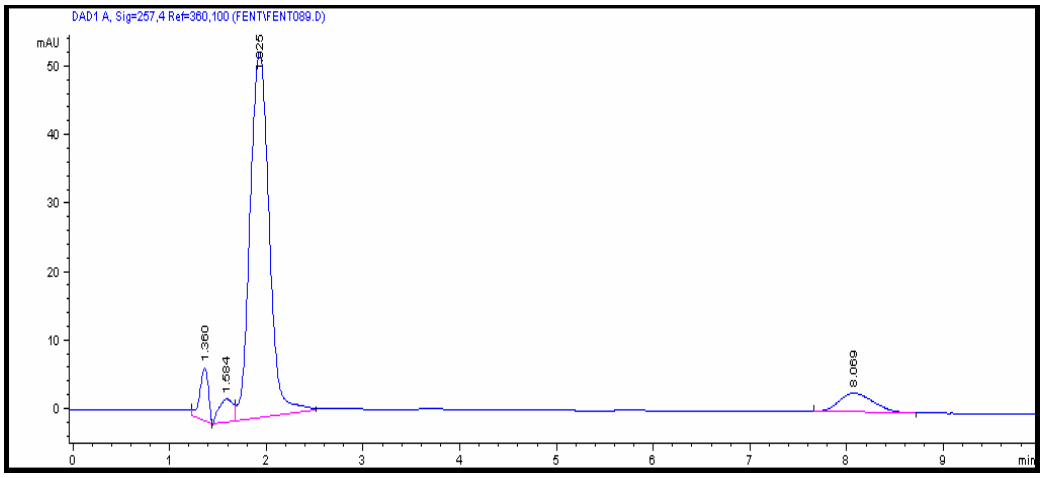


Figure -50: Chromatogram of fentanyl with mobile phase (60 %MeOH in acetate buffer pH 4.5).

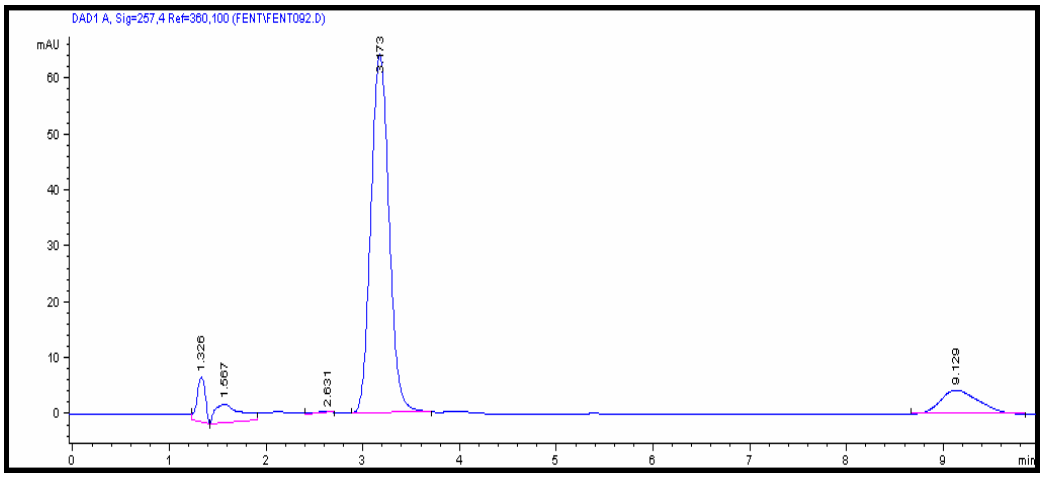


Figure -51: Chromatogram of fentanyl with mobile phase (60 %MeOH in acetate buffer pH 6.7).

7.2 Preliminary Assay Development on the LCQ

This preliminary development on the LCQ was done mainly to obtain some experience with LC/MS equipment before going on to operating a triple-quad LC/MS/MS instrument.

7.2.1 Instrumental Conditions

HPLC analysis was performed using an Agilent 1100 Series quaternary pump combined with an HP 1050 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). The column used was Discovery® C_{18} bonded 5 µ silica, (15 cm x 2.1 mm) (Supelco, USA) with a mobile phase flow rate of 0.3 ml/min. The detector was a Finnigan LCQ™ LC/MS^n System.

7.2.2 Preparation of Infusion Solutions

Fentanyl Infusion Solutions

- To 1ml of fentanyl citrate injection solution (50 µg/ml fentanyl equivalent) in a screw-capped glass extraction tube was added 100 µl of 10 M NaOH and 10 ml ethyl ether.
- The tube was shaken vigorously for 2 minutes.
- Centrifuged for 2 min at 2000 rpm and 4°C
- The aqueous phase was frozen in an alcohol freezing bath at −29°C
- Ethyl ether was decanted into another glass test tube.
- Ethyl ether was evaporated under a stream of nitrogen.
- The extract was reconstituted in 10 ml methanol to obtain a 5 µg/ml solution, which was stored refrigerated in a scintillation vial.
- 100 µl of the 5 µg/ml solution was transferred to another disposable glass ampoule and methanol was evaporated, and the residue reconstituted with 5 ml of the mobile phase (60 % MeOH in 0.05 M acetate buffer adjusted to apparent pH 4.5), to obtain a 100 ng/ml fentanyl solution.

D_5-Fentanyl Infusion Solution

D_5-fentanyl stock solutions at relevant concentrations of D_5-fentanyl were obtained by spiking the methanol solution obtained from Cerilliant™ into the respective solvents. In this case, 100 µl of a stock solution containing 1 µg/ml D_5-fentanyl in methanol was transferred into a disposable glass ampoule and the methanol evaporated. The residue was also reconstituted in 1 ml of the same mobile phase to obtain a 100 ng/ml solution.

7.2.3 Creating Tune Methods

Two tune methods (high.tun, and low.tun) for both fentanyl and D_5-fentanyl were created by setting the following acquisition parameters:

High.Tun Method	**Set**	**Actual**
Sheath gas flow rate (arb)	70	69.43
Aux. Gas flow rate (arb)	5	4.4
I spray Voltage [K.V]	4.25	4.23
Spray current(μA)		0.24
Capillary temperature [^{o}C]	200	199.9
Capillary Voltage [V]	20	19.98
Tube Lens offset [V]	10	

Note:

1. The parameters were the same for fentanyl and D_5-fentanyl.
2. The parameters for low.tun are the same as that of high.tun, except for the sheath gas flow rate, which is 40, and spray current 0.05 – 0.1(μA).

After setting the parameters, the infusion solutions (100 ng/ml fentanyl & 100 ng/ml D_5-fentanyl) were infused into the LCQ directly using a 250 μl syringe one at a time with a flow rate of 5 μl/minute. Fentanyl gave spectra of its molecular ion at m/z = 337.2 and daughter ion at m/z = 188, while D_5-fentanyl gave ions at m/z = 342 and 188, respectively.

7.3 LC/MS/MS

7.3.1 Instrumental and Chromatographic Conditions

The LC-MS/MS system used was a Sciex API 2000 system (Applied Biosystems, Ontario, Canada) with turbo-ion spray ionization in the positive ion mode. HPLC analysis was performed using an Agilent 1100 series quaternary pump combined with an HP 1100 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). Chromatography was performed on a Supelco Discovery® C_{18} (150 x 2.0 mm, 5 μm) stainless steel column. The mobile phase used was methanol: 0.05 M acetic acid (60:40 (v/v) pH adjusted to 4.54 using 25% ammonia solution) at a flow rate of 0.2 ml/min. A Hewlett Packard Series 1100 auto sampler equipped with a cooling device maintaining the temperature at 5°C was used to

inject 20 µl of the extracts onto the HPLC column.

7.3.2 Preparation of Infusion Solutions

500 µl of 5 µg/ml fentanyl solution in methanol (prepared in section 7.2.2) was pipetted into a 5 ml disposable glass ampoule and the methanol evaporated. The residue was reconstituted in 5 ml of mobile phase; (MeOH: 0.05M AcOH; 60:40 (v/v) adjusted to pH 4.5), to obtain a 500 ng/ml fentanyl solution.

Similarly 2.5 ml of 1 µg/ml D_5-fentanyl solution in methanol was evaporated, and the residue was reconstituted in 5 ml mobile phase to obtain a 500 ng/ml D_5-fentanyl solution.

These solutions were then infused into the LC/MS/MS system using a 250 µl syringe at a flow rate of 10 µl/min.

Infusion quantitative optimization

This was performed in creating an acquisition method:

MS/MS analysis	Positive
Precursor ion	336.5 for fentanyl and 341.5 for D_5-fentanyl
Search window	±1.000(amu)
Product ion:	selected automatically from the 6 most intense peaks including the precursor ion

Both quad 1 & quad 3 were set on unit resolution.

Target compound	Optimum Acquisition Parameters Selected by Instrument during Auto tune							
	Mass (amu)	No of Charges	DP	FP	EP	CEP	CE	CXP
Fentanyl	337.5/187.9	1	21	370	11.5	18	33	10
D_5-fentanyl	342.5/187.9	1	21	370	11.5	20	33	10

The instrument's automatic optimisation algorithm found the parent ions (M+1) m/z = 337 for fentanyl and 342 for D_5-fentanyl and optimized the acquisition parameters for the product ion m/z = 188 for both fentanyl and D_5-fentanyl. (See Figures - 52 to 55).

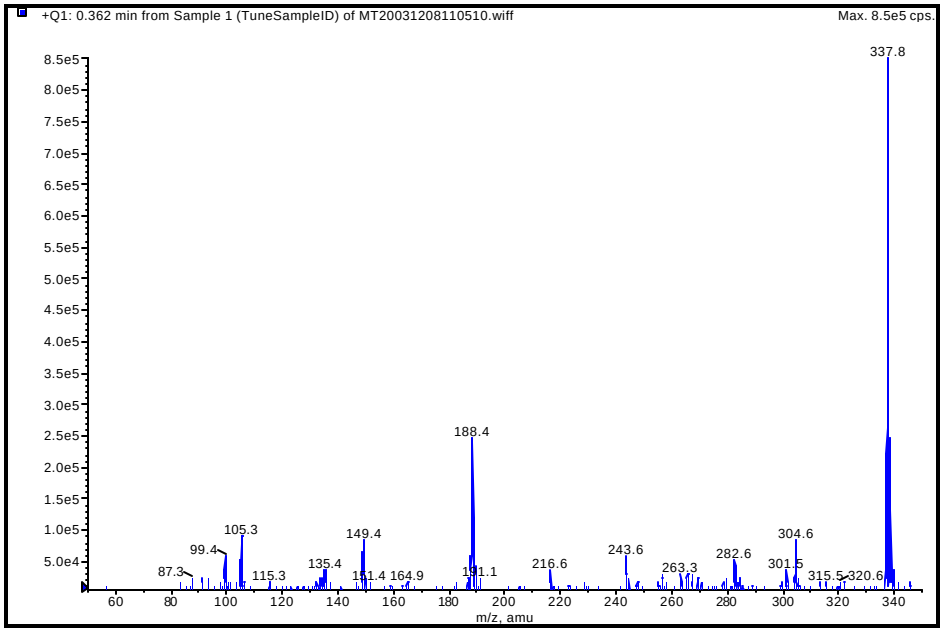


Figure -52: Infusion mass spectrum of fentanyl

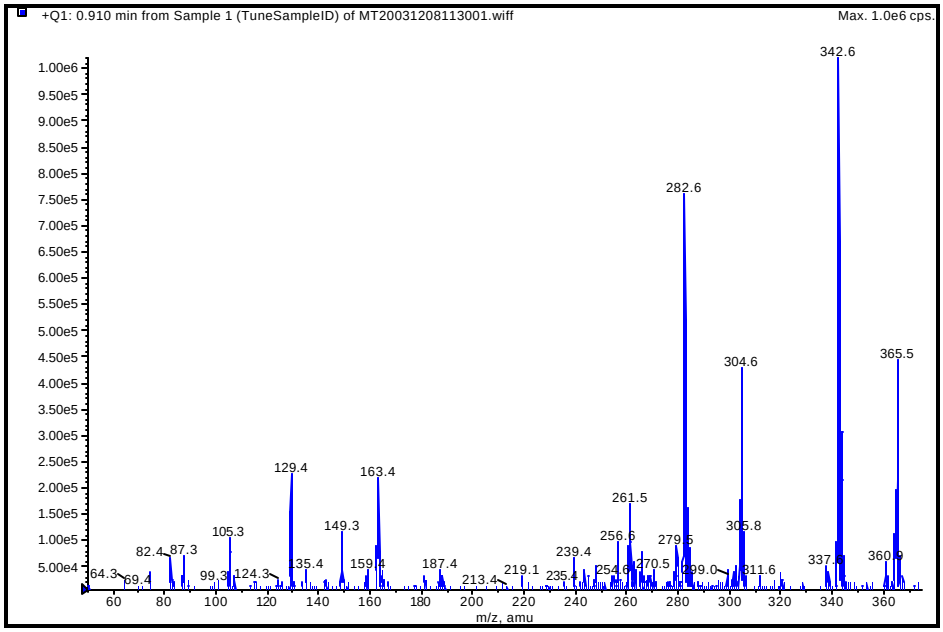


Figure 53: Infusion mass spectrum of D_5-fentanyl

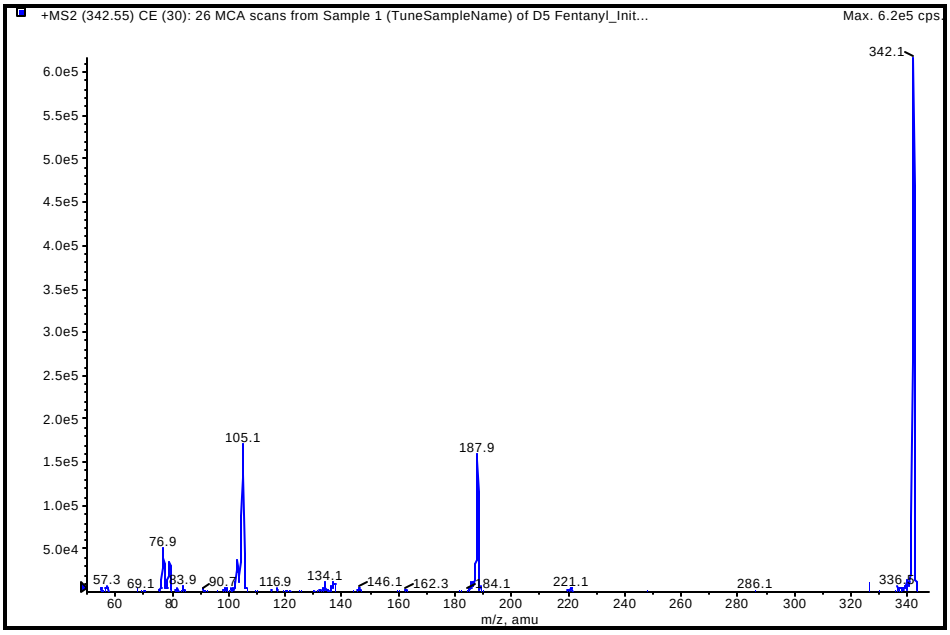


Figure -54: Infusion product ion mass spectrum of D_5-fentanyl (parent ion m/z = 342)

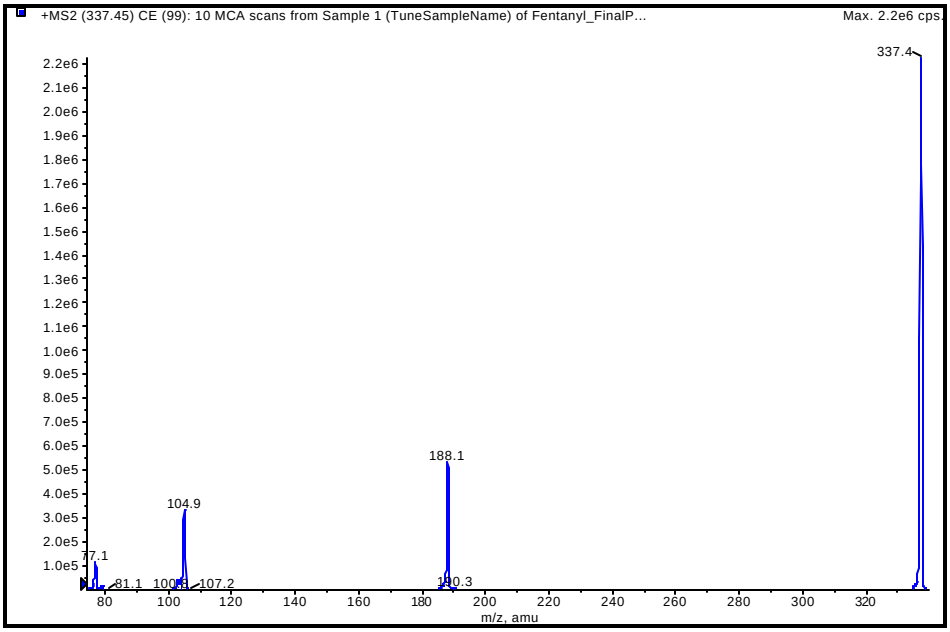


Figure 55: Infusion product ion mass spectrum of fentanyl (parent ion m/z = 337)

7.3.3 Preparation of System Performance Verification Standard (SPVS)

400 µl of 5 µg/ml fentanyl solution in methanol, and 2 µl of 1 µg/ml D_5-fentanyl solution in methanol were pipetted into two 10 ml ampoules. The methanol was evaporated and the residues were reconstituted with 10 ml of 2 % formic acid to obtain a 200 ng/ml of each component in solution. 10 ml of each solution were then mixed to obtain an SPVS solution containing 100 ng/ml of each of the analytes.

7.3.4 Detector Response Consistency and Linearity Test

To check the on-instrument reproducibility (consistency of response) 0.5 ml of the SPVS was transferred into an autosampler injection vial, and a sequence of 50 x 20 µl injections made onto the column. The flow rate of the mobile phase was 200 µl/min.

Together with the consistency test, a response linearity test was also performed using the SPVS. 750 µl SPVS was pipetted into a 5 ml disposable glass ampoule and serially diluted (1:1) with 2 % formic acid down to 0.19 ng/ml, and run in the same batch sequence.

The mean results are presented in Table-35 and the complete results in Figure-56.

Table-35: Summary of on-instrument reproducibility data; using SPVS

	Fentanyl	D_5-Fentanyl	Ratios
Mean Peak Area	167718	149024	1.09
Std. Dev	18113.22	14210.12	1.12
% CV	10.8	9.5	1.7

The % CV of 10.8 & 9.5 respectively, for fentanyl and D_5-fentanyl peak area, shows that the instrument response is consistent (reproducible).

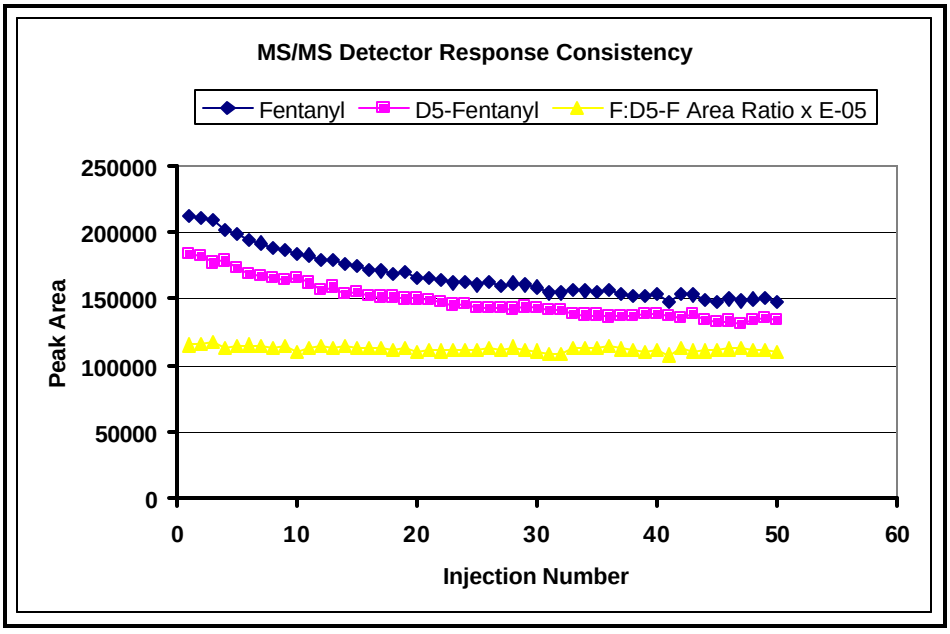


Figure-56: Detector response consistency

In the consistency test, peak areas decreased steadily (from ~210,000 to 160,000) for the first 20 injections, but after that it stabilized.

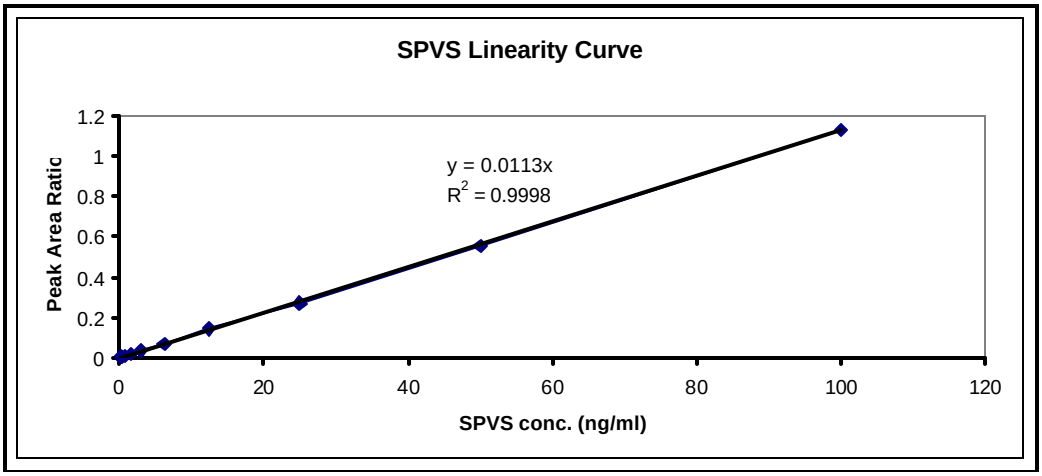


Figure -57: SPVS linearity curve

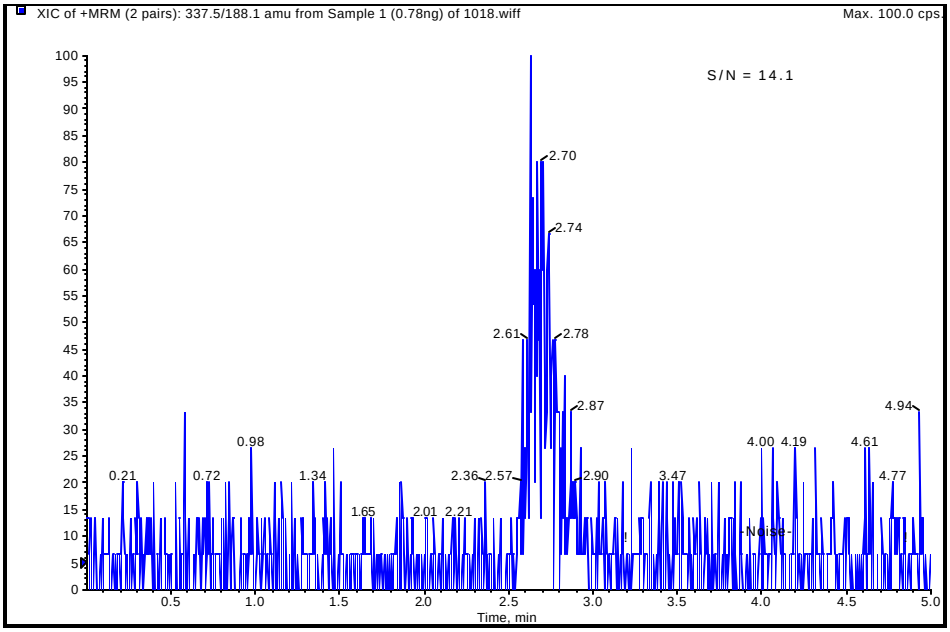


Figure -58: SPVS chromatogram at estimated LLOQ (0.78 ng/ml)

7.3.5 Extraction Consistency and Linearity Test

50 ml (51.345 g) of normal plasma was spiked with 100 µl of fentanyl citrate injection solution (50 µg/ml fentanyl equivalent) to obtain 100 ng/ml fentanyl-plasma standard (STD A).

For the consistency test, aliquots (1 ml) of 100 ng/ml fentanyl plasma standards were pipetted into 14 disposable 10 ml glass ampoules.

For the linearity test 4 ml of 100 ng/ml (STD A) was pipetted into a scintillation vial and was serially diluted (1:1) with normal plasma to obtain 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78, 0.39, and 0.19 ng/ml fentanyl plasma standards. Then they were aliquoted (1ml) in to 10 ml disposable glass ampoules.

The 24 plasma samples were extracted as follows:

- Add 100 µl of D_5-fentanyl solution (1 µg/ml in 0.01N HCl)
- Add 100 µl of 10 M NaOH, and 5 ml ethyl ether
- Vortex mix for 2 min.
- Centrifuge at 2000 rpm and 4°C for 2 min.
- Freeze aqueous phase in alcohol freezing bath at –29°C
- Decant ethyl ether into 5 ml ampoules containing 500 µl of 2 % formic acid
- Vortex mix for 2 minutes
- Centrifuge at 2000 rpm and 4°C for 2 min.
- Freeze aqueous phase in alcohol freezing bath at –29°C
- Discard the ether layer
- Evaporate residual ether in ampoule at low temperature (~30°C), under a flow of nitrogen for 1 min.
- Transfer extracts to autosampler vials with micro inserts
- Inject 10 µl onto the column.

Before injecting the consistency and linearity samples, 25 injections of SPVS extracts were done.

The results are summarised in Table-36.

Table-36: Summary of plasma (100 ng/ml) extraction consistency

	Fentanyl		D_5-Fentanyl		Area Ratio
	Peak Area	Rt	Peak Area	Rt	
	197700	2.67	194300	2.64	1.02
	212100	2.65	201000	2.64	1.06
	232500	2.67	206900	2.65	1.12
	211000	2.68	205400	2.65	1.03
	226700	2.67	214200	2.65	1.06
	207700	2.65	190500	2.64	1.09
	218900	2.66	215500	2.66	1.02
	207400	2.68	204600	2.65	1.01
	228700	2.65	201600	2.65	1.13
	193700	2.67	189000	2.64	1.03
	200900	2.68	203000	2.64	0.99
	222600	2.67	209900	2.64	1.06
	192200	2.67	207100	2.64	0.93
	204600	2.66	198800	2.65	1.03
Mean	211192.86	2.67	202985.71	2.65	1.04
Std Dev.	13056.47	0.01	7947.80	0.01	0.05
CV %	6.18	0.41	3.92	0.24	5.11

The results clearly demonstrated excellent extraction reproducibility

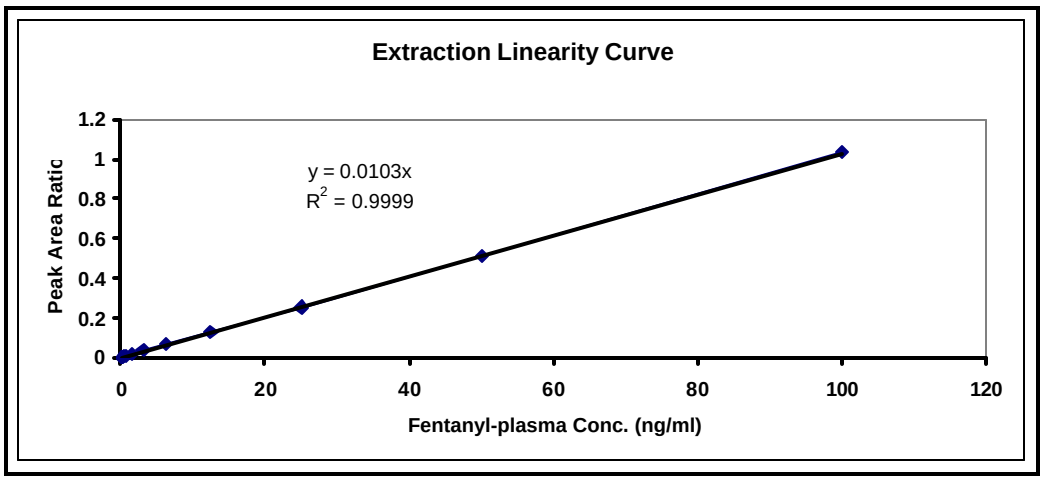


Figure -59: Extraction linearity curve

The volume of 2 % formic acid used for back-extraction in the above experiment was 500 µl, and the volume injected 10 µl. To increase the sensitivity of the assay method the volume of the formic acid was decreased to 250 µl, and the volume injected increased to 20 µl. Then the linearity test was repeated and gave a linear calibration curve with r = 0.9999. Sensitivity improved, and the LLOQ achievable was estimated to be about 0.3 ng/ml.

7.3.6 Matrix Effect

It has been noted that co-eluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LC/MS-MS assay. In order to determine whether this effect (called the matrix effect) is present or not, 10 different plasma pools were extracted and each extract spiked with known concentrations of fentanyl and D_5-fentanyl. These samples were injected and peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect. Though matrix effects cannot be excluded as an ion suppression factor, the effect did not influence the reproducibility of this method as shown by the data in Table-37.

Table-37: Matrix effect

Plasma pool	Fentanyl		D_5-Fentanyl (ISTD)		Ratio	
	Area of 100ng/ml	Area of 20ng/ml	Area of 100ng/ml	Area of 20ng/ml	Area of 100ng/ml	Area of 20ng/ml
1	857500	183900	647800	176500	1.3237	1.0419
2	926600	200100	714200	186300	1.2974	1.0741
3	885500	204100	680900	190900	1.3005	1.0691
4	855600	206300	657800	190700	1.3007	1.0818
5	824800	207400	643300	196100	1.2821	1.0576
6	880400	177200	687600	172200	1.2804	1.0290
7	909000	213600	695700	195400	1.3066	1.0931
8	836400	179200	640700	166300	1.3054	1.0776
9	891400	203800	687200	189100	1.2971	1.0777
10	858800	182000	670300	169700	1.2812	1.0725
Average	872600	195760	672550	183320	1.2975	1.0675
STDEV	31841	13608	24691	11100	0.0135	0.0194
%CV	3.65	6.95	3.67	6.05	1.04	1.81

The low % CV for the peak areas of the spiked extracts indicate that the extracts contained no co-eluting compounds adversely affecting the ionization of fentanyl.

8 VALIDATION OF THE LC/MS-MS ASSAY METHOD

8.1 Instrumental and Chromatographic Conditions

The LC-MS / MS system used was a Sciex API 2000 system (Applied Biosystems, Ontario, Canada) with turboion spray ionization in the positive ion mode. HPLC analysis was performed using an Agilent 1100 Series quaternary pump combined with an HP 1100 series autosampler, and an Agilent 1100 series vacuum degasser (Germany). Chromatography was performed on a Supelco Discovery® C18 (150 x 2.0 mm, 5 µm) stainless steel column. The mobile phase used at a flow rate of 0.2 ml/min., was methanol: 0.05 M acetic acid (60:40(v/v)) pH adjusted to 4.54 using a 25% ammonia solution. A Hewlett Packard Series 1100 auto sampler equipped with a cooling device maintaining the temperature at 5°C, was used for injecting an aliquote of 20 µl of the extracts onto the HPLC column.

Table - 38: Ionisation source settings

Curtain Gas	20.0
Collision Gas	4.0
Ion Spray Voltage (V)	5000.0
Heated Nebulizer (°C)	400.0
Ion Source Gas1 (GS1)	70.0
Ion Source Gas 2 (GS2)	70.0

Table -39: MS-MS detector settings

	Fentanyl	D5-fentanyl
Monoisotopic Molecular Mass	336.500	341.500
Protonated Molecular Ion (m/z)	337.450	342.550
Dwell Time (ms)	150.000	150.000
Product Ion (m/z)	188.150	188.150
Declustering Potential (V)	21.0	21.0
Focusing Potential (V)	370.0	370.0
Entrance Potential (V)	11.5	11.5
Collision Cell Entrance Potential (V)	18.0	20.0
Collision Energy (eV)	33.0	33.0
Collision Cell Exit Potential (V)	10.0	10.0
Scan Type	MRM	MRM
Polarity	Positive	Positive
Pause Time	5ms	5ms

8.2 Extraction Procedure

The plasma samples were completely thawed unassisted at room temperature. They were then vortexed for 1 min., followed by centrifugation for five minutes at 5000 rpm to remove cryo proteins that had formed during the storage.

- Plasma samples (1 ml) were pipetted into 10 ml disposable glass ampoules.
- 100 µl of a solution of 625 ng/ml D_5-fentanyl (ISTD) in 0.01N HCl, 100 µl of 10 M NaOH, and 5 ml of ethyl ether were added, and the sample vortex mixed for 2 min.
- After centrifugation for 2 min. at 2000 rpm and 4ºC, the aqueous phase was frozen in alcohol freezing bath at –29ºC.
- The organic phase was decanted into 5 ml ampoules containing 250 µl of 2 % formic acid, and vortex mixed for 2 min. followed by centrifugation for 2 min. at 2000 rpm and 4ºC.
- The aqueous phase was frozen, and the organic phase discarded.

- The aqueous phase (extracts) was evaporated at low temperature (~30°C) under a flow of N_2 gas for 1 min. to remove any remaining ethyl ether followed by vortexing for 30 sec.
- The extracts were transferred into the autosampler injection vials containing micro glass inserts, and 20 µl injected onto the column.

8.3 Preparation of Plasma Calibration Standards

The following three phases in the validation process required calibration and quality control standards

- Intra-day validation
- Inter-day-I validation
- Inter-day-II validation.

Based on the number of calibration standards, the volume of plasma required for the validation was calculated with the aid of a calculation sheet set up in Excel (Table -40).

Table -40: Calculated volume of plasma needed for preparation of STDs and QCs required in validation.

Phase	Sets of STDs (A)	Sets of QCs (B)	Levels STDs (C)	Levels QCs (D)	Replicates No. STDs(E)	Replicates QCs(F)	Sample volume (G) in ml	Volume(ml)
Intra-day	1	1	10	6	2	9	1.2	88.8
Inter-day-I	1	1	9	6	1	8	0.8	45.6
Inter-day-II	1	1	9	6	1	8	0.8	45.6
							Total	180

Volume of plasma = A x C x E x G +B x D x F x G

The maximum concentration (Cmax) was expected to be ~78 ng/ml, and the attainable LLOQ estimated to be 0.30 ng/ml. It was therefore decided to validate the assay method from 2 x Cmax (STD K = ~155 ng/ml) down to the LLOQ (STD B = ~0.30 ng/ml).

A stock solution of 181.63 µg/ml fentanyl in water was prepared by dissolving 3.110 mg of fentanyl citrate salt (Sigma-Aldrich. F3886), equivalent to 1.980 mg free base, in 10.90 g of water as indicated in Table -41.

A pool of normal plasma (STD K) was spiked with 100 µl of the stock solution and serially diluted (1:1) with normal plasma to obtain the required concentrations (Table -42). STD J

represents Cmax with a concentration of 77.60 ng/ml, while STD B (0.303 ng/ml) the LLOQ. The calibration standards were aliquoted (1.2 ml) into polypropylene tubes and stored at ~-20°C.

Table -41: Preparation of stock solution for spiking STD K

Solvent Used	SG Solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Volume Spiked (µl)	Concentration Analyte (µg/ml)
Water	1.000	1.980	10.900	10.900	100	181.63

Table -42: Preparation of plasma calibration standards

Sample Code & No.	Source Solution	A	B	C	D ng/ml
STD K	**Stock SA**	46.102	166.104		155.29
STD J	**STD K**	46.044	106.050	166.056	77.65
STD I	**STD J**	45.996	105.998	166.016	38.83
STD H	**STD I**	45.385	105.388	165.400	19.42
STD G	**STD H**	44.494	104.497	164.515	9.71
STD F	**STD G**	45.575	105.572	165.564	4.85
STD E	**STD F**	45.752	105.749	165.754	2.43
STD D	**STD E**	45.422	105.426	165.427	1.21
STD C	**STD D**	44.664	104.662	164.662	0.61
STD B	**STD C**	44.575	104.584	164.587	0.30

Note: Mass of biological fluid (g) is converted to volume (ml). SG = 1.0269Kg/L for plasma

KEY: A = Mass of empty container, B = Mass of container and normal plasma, C = Total mass of container + normal + spiked plasma, D = concentration (ng/ml).

8.4 Preparation of Plasma Quality Control Standards (QCs)

A stock solution of 166.37 µg/ml fentanyl in water was prepared by dissolving 3.29 mg fentanyl citrate salt (F3886), equivalent to 2.094 mg fentanyl free base, in 12.589 g of water as indicated in Table-43. A pool of normal plasma (QC I) was spiked with 100 µl of the stock solution and serially diluted (1:1) with normal plasma to obtain the required concentrations (Table-44). The QCs were aliquoted (1.2 ml) into polypropylene tubes and stored at ~-20°C together with the calibration standards.

Table-43: Preparation of stock solution for spiking QC I

Solvent Used	SG Solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Volume Spiked(µl)	Concentration Analyte (µg/ml)
Water	1.000	2.094	12.589	12.589	100	166.37

Table-44: Preparation of plasma quality control standards (QCs)

Sample Code & No.	Source Solution	A	B	C	D ng/ml
QC I	**Stock QA**	0.000	129.980		131.34
QC H	**QC I**	45.990	111.020	176.060	65.67
QC G	**QC H**	45.856	110.830	175.866	32.85
QC F	**QC G**	44.540	109.530	174.522	16.43
QC E	**QC F**	44.714	109.746	174.764	8.21
QC D	**QC E**	44.680	124.600	174.630	3.16
QC C	**QC D**	44.587	109.600	175.670	1.59
QC B	**QC C**	44.570	109.560	174.580	0.80
QC A	**QC B**	44.550	109.560	174.560	0.40

Note: Mass of biological fluid (g) is converted to volume (ml). SG = 1.0269Kg/L for plasma

KEY: A = Mass of empty container, B = Mass of container and normal plasma, C = Total mass of container + normal + spiked plasma, D = concentration (ng/ml).

8.5 Intra-batch Accuracy and Precision

The method was validated by analyzing plasma quality control samples six times at nine different concentrations to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve with ten different concentrations from 0.30 to 155 ng/ml. Calibration graphs were constructed using several regression types of the analyte peak area vs nominal drug concentration, and the best curve fit was obtained using a linear regression weighted $1/c^2$.

Intra-batch accuracy and precision were assessed by the assay of all calibration standards in duplicate to produce one calibration curve and six replicates of all the prepared QCs in a single batch of assays. The intra-batch accuracy and precision of the assay procedure were assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas to get two different quantitation methods.

Accuracy is expressed as recovery of the analyte as % nominal while the precision is expressed as the CV %. For a valid method the intra-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nominal should be between 85 % and 115 %) over most of the range, and within 20 % of nominal concentration at the LLOQ. For a valid method the intra-batch precision is required to be less than 15 % (i.e. CV % should be less than 15 %) over most of the range, and less than 20 % at the LLOQ.

The method performed well using both quantitation methods (peak height and peak area). The peak area quantitation method gave the best results and was used for the statistical analysis of the two inter-batch validations.

The results of the intra-batch validation are summarized in Tables 45 & 46 for quantitation by peak height, and in Tables 47 & 48 for the quantitation by peak area.

8.5.1 Quantitation by Peak Height Ratios

Table - 45: Back calculated concentrations of fentanyl based on peak height ratios

STD Code	Nominal Concentration (ng/ml)	Back-calculate d Concentration (ng/ml)	% Dev
STD K	155	156.492	1.0
STD K	155	160.378	3.5
STD J	77.6	75.587	-2.6
STD J	77.6	71.698	-7.6
STD I	38.8	36.052	-7.1
STD I	38.8	34.771	-10.4
STD H	19.4	20.484	5.6
STD H	19.4	18.691	-3.7
STD G	9.71	10.892	12.2
STD G	9.71	10.231	5.4
STD F	4.85	5.275	8.8
STD F	4.85	4.943	1.9
STD E	2.43	2.425	-0.2
STD E	2.43	2.306	-5.1
STD D	1.21	1.196	-1.2
STD D	1.21	1.288	6.4
STD C	0.607	0.548	-9.7
STD C	0.607	0.604	-0.5
STD B	0.303	0.329	8.7
STD B	0.303	0.287	-5.3

Quantification Method = Peak height ratio, Regression Equation = Linear $1/C^2$ (slope = 0.015599, intercept = 0.001726, $R^2 = 0.994281$)

Table-46: Summary of intra-batch quality control results based on peak height ratios

Code Nominal / Replicates	QC I 131.00 ng/ml	QC I dil 131.00 ng/ml	QC H 65.70 ng/ml	QC G 32.90 ng/ml	QC F 16.40 ng/ml	QC E 8.21 ng/ml	QC D 3.16 ng/ml	QC C 1.59 ng/ml	QC B 0.80 ng/ml	QC A 0.40 ng/ml
1	129.49	140.65	78.13	35.71	16.44	9.87	3.26	1.82	0.82	0.37
2	141.70	134.23	67.51	32.41	16.95	8.44	3.00	1.57	0.80	0.35
3	141.22	118.13	68.52	33.92	16.92	7.96	3.20	1.54	0.80	0.37
4	129.88	129.61	60.29	32.19	18.53	8.44	3.43	1.71	0.90	0.40
5	148.49	128.55	64.00	31.94	17.17	8.78	3.28	1.50	0.81	0.44
6	143.51	127.99	69.18	32.96	17.83	8.43	3.13	1.56	0.76	0.44
MEAN	139.05	129.86	67.94	33.19	17.31	8.65	3.22	1.62	0.81	0.39
%nom	106.1	99.1	103.4	100.9	105.5	105.4	101.8	101.8	102.2	98.9
CV%	5.5	5.7	8.8	4.3	4.4	7.5	4.5	7.6	5.6	9.9

8.5.2 Quantitation by Peak Area Ratios

Table-47: Back calculated concentrations of fentanyl based on peak area ratios

STD Code	Nominal Concentration (ng/ml)	Back-calculated Concentration (ng/ml)	% Dev
STD K	155	155.677	0.4
STD K	155	160.870	3.8
STD J	77.6	74.420	-4.1
STD J	77.6	72.701	-6.3
STD I	38.8	37.180	-4.2
STD I	38.8	35.353	-8.9
STD H	19.4	20.377	5.0
STD H	19.4	18.707	-3.6
STD G	9.71	10.981	13.1
STD G	9.71	10.052	3.5
STD F	4.85	5.145	6.1
STD F	4.85	4.969	2.4
STD E	2.43	2.338	-3.8
STD E	2.43	2.399	-1.3
STD D	1.21	1.263	4.4
STD D	1.21	1.250	3.3
STD C	0.607	0.569	-6.2
STD C	0.607	0.550	-9.3
STD B	0.303	0.344	13.4
STD B	0.303	0.279	-7.9

Quantification Method = Peak area ratio, Regression Equation = Linear $1/C^2$ (slope = 0.015686, intercept = 0.000901, $R^2 = 0.994003$)

Table-48: Summary of intra-batch quality control results based on peak area ratios

Code Nominal / Replicates	QC I 131.00 ng/ml	QC I dil 131.00 ng/ml	QC H 65.70 ng/ml	QC G 32.90 ng/ml	QC F 16.40 ng/ml	QC E 8.21 ng/ml	QC D 3.16 ng/ml	QC C 1.59 ng/ml	QC B 0.80 ng/ml	QC A 0.40 ng/ml
1	134.30	143.29	78.40	35.09	16.19	9.45	3.15	1.73	0.86	0.42
2	141.12	131.56	69.75	32.66	16.77	8.33	3.06	1.66	0.80	0.35
3	144.30	122.01	66.10	34.23	17.45	7.99	3.11	1.56	0.75	0.37
4	134.56	124.47	60.06	32.38	18.86	8.43	3.37	1.65	0.87	0.37
5	148.64	129.75	63.76	32.21	17.03	8.53	3.20	1.45	0.76	0.39
6	144.07	127.74	69.21	33.58	17.54	8.27	2.99	1.54	0.81	0.41
MEAN	141.16	129.80	67.88	33.36	17.31	8.50	3.15	1.60	0.81	0.38
%nom	107.8	99.1	103.3	101.4	105.5	103.5	99.6	100.5	101.4	96.3
CV%	4.1	5.7	9.3	3.4	5.2	5.9	4.2	6.2	6.1	6.9

8.6 Inter-batch Accuracy and Precision

Inter-batch accuracy and precision are assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards (from Cmax to LLOQ) and six replicates of each of the quality control standards (i.e. from highest, medium, and lowest concentrations) designated for use in the assay of samples of unknown concentrations. Five levels of QCS were used. These are:

Highest 1.9 Cmax (QC H)

High 0.8 Cmax (QC G)

Medium 0.5 Cmax (QC E)

Low 2.3 x LLOQ (QC B)

LLOQ 1.2 – 1.8 x STD B (QC A)

The inter-batch accuracy and precision of each of the batches is assessed separately by calculating the regression equation and constructing the calibration curve based on the best performing method and must pass the criteria for inter-batch acceptance. The inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 validations). Accuracy is expressed as the % difference between the nominal and calculated value or as % nominal

of the analyte, while precision is expressed as the coefficient of variation (% CV). For a valid method the intra- and inter-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of the nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (i.e. % CV should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the two inter-batch validations with highest variation 11.7 % (QC A in inter-batch1).

The results are summarized in tables 49,50,51, & 52.

8 6.1 Inter-batch 1 Accuracy and Precision

Table -49: Back calculated concentrations of fentanyl based on peak area ratio

STD Code	Nominal Concentration (ng/ml)	Back-calculated Concentration (ng/ml)	% Dev
STD J	77.600	78.998	1.8
STD I	38.800	36.557	-5.8
STD H	19.400	20.461	5.5
STD G	9.710	9.309	-4.1
STD F	4.850	4.920	1.4
STD E	2.430	2.374	-2.3
STD D	1.210	1.253	3.6
STD C	0.607	0.596	-1.9
STD C	0.607	0.625	2.9
STD B	0.303	0.309	2.0
STD B	0.303	0.293	-3.2

Quantification Method = Peak area ratio, Regression Equation = Linear $1/C^2$ (slope = 0.146215, intercept = 0.006399, $R^2 = 0.997934$)

Table -50: Summary of quality control results for inter-batch 1 validation

Code Nominal / Replicates	QC H 65.7 ng/ml	QC G 32.9 ng/ml	QC E 8.21 ng/ml	QC B 0.80 ng/ml	QC A 0.40 ng/ml
1	73.14	31.39	7.67	0.77	0.35
2	65.75	32.76	8.93	0.80	0.34
3	67.08	31.77	8.37	0.87	0.35
4	70.57	31.79	7.99	0.71	0.31
5	71.36	36.80	8.42	0.81	0.40
6	71.52	35.90	8.24	0.84	0.43
MEAN	69.90	33.40	8.27	0.80	0.36
%nom	106.4	101.5	100.7	100.5	91.2
CV%	4.1	7.0	5.2	6.8	11.7

8 6.2 Inter-batch 2 Accuracy and Precision

Table -51: Back calculated concentrations of fentanyl based on peak area ratio

STD Code	Nominal Concentration (ng/ml)	Back-calculated Concentration (ng/ml)	% Dev
STD J	77.600	78.589	1.3
STD I	38.800	37.874	-2.4
STD H	19.400	20.397	5.1
STD G	9.710	9.828	1.2
STD F	4.850	4.737	-2.3
STD E	2.430	2.440	0.4
STD D	1.210	1.109	-8.3
STD C	0.607	0.609	0.4
STD C	0.607	0.640	5.5
STD B	0.303	0.324	6.8
STD B	0.303	0.280	-7.7

Quantification Method = Peak area ratio, Regression Equation = Linear $1/C^2$ (slope = 0.158386, intercept = 0.004024, R^2 = 0.996088)

Table -52: Summary of quality control results for inter-batch 2 validation

Code Nominal	QC H 65.7 ng/ml	QC G 32.9 ng/ml	QC E 8.21 ng/ml	QC B 0.80 ng/ml	QC A 0.40 ng/ml
Replicates					
1	67.82	31.82	8.28	0.82	0.40
2	63.56	31.51	8.65	0.78	0.39
3	66.35	32.31	7.95	0.80	0.38
4	66.25	27.13	8.51	0.74	0.38
5	66.08	32.58	7.85	0.77	0.37
6	65.90	34.35	8.45	0.78	0.41
MEAN	65.99	31.62	8.28	0.78	0.39
%nom	100.4	96.1	100.9	98.1	97.6
CV%	2.1	7.6	3.9	3.4	3.5

8.7 Summary of The Combined Quality Control Results for The 3 Validations

The combined quality control results are summarized in Table-53. The method performed well during the course of all the three validations with the highest CV of 8.2 % for QC A.

Table-53: Summary of the combined quality control results of the 3 validations

	Nominal Conc. (ng/ml)	QC H 66ng/ml	QC G 33ng/ml	QC E 8ng/ml	QC B 1ng/ml	QC A 0.4ng/ml
	Replicates					
Validation Batch1	**1**	78.400	35.090	9.450	0.860	0.420
	2	69.750	32.660	8.330	0.800	0.350
	3	66.100	34.230	7.990	0.750	0.370
	4	60.060	32.380	8.430	0.870	0.370
	5	63.760	32.210	8.530	0.760	0.390
	6	69.210	33.580	8.270	0.810	0.410
Validation Batch 2	**1**	73.140	31.390	7.670	0.770	0.350
	2	65.750	32.760	8.930	0.800	0.340
	3	67.080	31.770	8.370	0.870	0.350
	4	70.570	31.790	7.990	0.710	0.310
	5	71.360	36.800	8.420	0.810	0.400
	6	71.520	35.900	8.240	0.840	0.430
Validation Batch 3	**1**	67.820	31.820	8.280	0.820	0.400
	2	63.560	31.510	8.650	0.780	0.390
	3	66.350	32.310	7.950	0.800	0.380
	4	66.250	27.130	8.510	0.740	0.380
	5	66.080	32.850	7.850	0.770	0.370
	6	65.900	34.350	8.450	0.780	0.410
	MEAN	67.926	32.807	8.351	0.797	0.379
	CV%	6.1	6.4	4.9	5.6	8.2
	%nom	103.4	99.7	101.7	99.6	94.7
	N	18	18	18	18	18

8.8 Stability Assessment

8.8.1 On-Instrument Stability

Sixteen stability samples of the same concentration were extracted, and then the extracts were combined and re-aliquoted (1.2 ml). They were injected in accordance with the intra- and first inter-batch validation run-sheets during the first two validation batches. Results are summarized in Table-54.

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Table-54: Stability data of sixteen STAB samples injected at different intervals

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area	IS Peak Area	Peak Area Ratio
1	05:21		0.00	522100	478500	1.091
2	06:32	01:11	1.18	585900	518100	1.131
3	07:48	01:15	2.45	397600	397200	1.001
4	08:59	01:11	3.63	377600	368400	1.025
5	10:20	01:21	4.98	357400	348200	1.026
6	11:42	01:21	6.34	394000	356400	1.105
7	13:08	01:25	7.78	491100	422100	1.163
8	13:13	00:05	7.86	487400	413500	1.179
9	05:30	16:17	72.14	521200	518200	1.006
10	06:16	00:46	72.91	618400	575800	1.074
11	07:06	00:50	73.75	428800	448800	0.955
12	07:57	00:50	74.60	377200	384900	0.980
13	08:48	00:50	75.45	357300	361700	0.988
14	09:39	00:50	76.30	396800	367500	1.080
15	10:35	00:55	77.23	423600	368400	1.150
16	10:40	00:05	77.31	412600	366500	1.126
			Mean	446812.50	418387.50	1.07
			Std Dev	81235.23	69805.35	0.07
			CV	18.18%	16.68%	6.68%

By regression analysis of the peak areas against the cumulative time data given in Table-54 it can be observed that the fentantyl peak area tends to decrease by 3.37% over a period of 77 hrs while staying on-instrument for injection (see Figure-60).

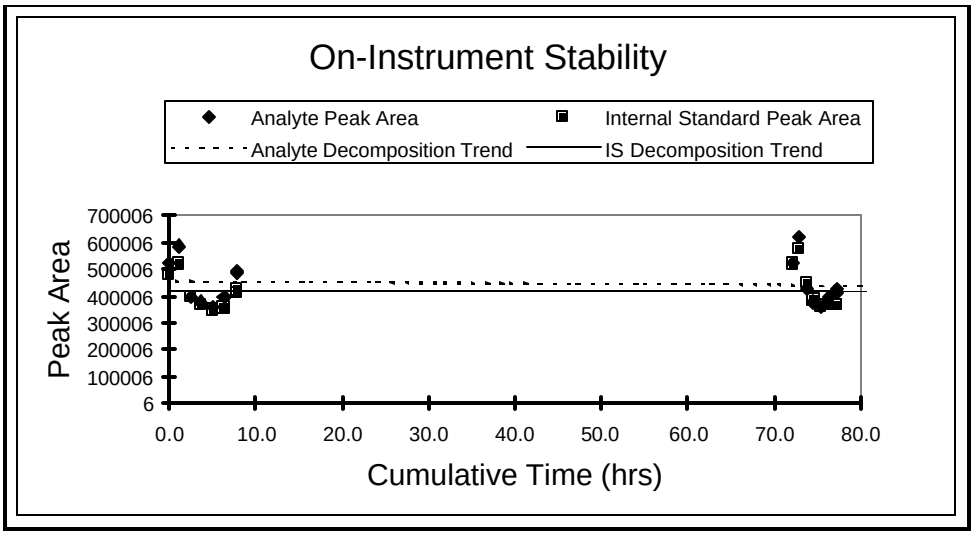


Figure -60: On-instrument stability chart

	Rate	Intercept	%/hr	%/Batch	Max Batch Duration
Decomposition Trend (Analyte):	-198.06	454660	-0.04%	-3.37%	344.33
Decomposition Trend (IS):	75.9192	415380	0.02%	1.41%	820.70
Decomposition Trend (Ratio):	-0.0006	1.0904	-0.05%	-4.09%	283.28

By regression analysis of the peak areas against the cumulative time data given in Table-54 it can be observed that the fentanyl peak area tends to decrease by 3.37% over a period of 77 hrs. while standing in the autosampler on-instrument for injection (see Figure-60).

The cyclic nature of the data demonstrated when sample extracts are injected at short time intervals for a period of about eight hours and repeated again on the same samples which had been left on the autosampler for another 64 hours is remarkable and illustrates the difficulties encountered in the determination of on-instrument stability with a detector that seems to be affected by the number of samples and the rate at which they are injected and eluted from the chromatography column into the detector.

This is probably a special case of the matrix effect that occurs as accumulated late eluting components elute from the column and has been observed in this laboratory on several occasions. The upward trend following an initial downward trend of the response shown in the first cycle of injections illustrates that the matrix effect is not one of ionisation suppression only but could also be responsible for ionisation enhancement. The fact that the response of the detector to the same samples injected about 64 hours later is practically the

same as for the first sequence of samples injected, illustrates unusual stability of the instrument and excellent on-instrument stability of fentanyl in the mobile phase. These data also stress the utility of using isotope-labelled internal standards for LC/MS assays whenever they are available.

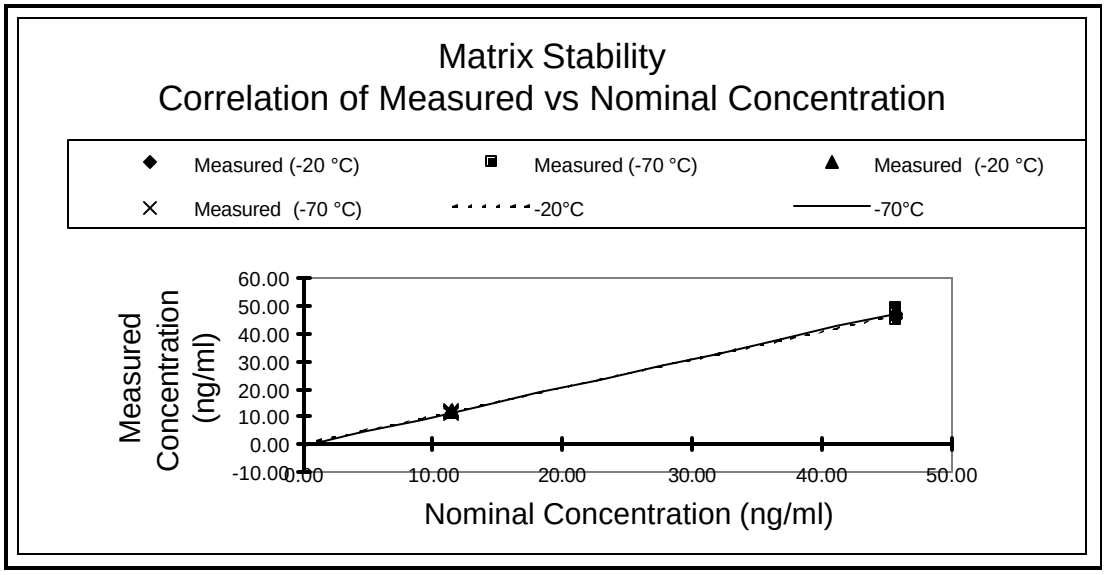
8.8.2 Stability in Matrix (Long-Term Stability)

For the determination of long-term stability, fentanyl spiked plasma standards at two different concentrations (45.74 & 11.40 ng/ml) were stored at -20^{o}C and -70^{o}C for 100 days. These samples were then assayed together with a freshly prepared set of calibration and quality control standards. The results of the measured concentrations of the stability samples are summarized in Table-55.

Table-55: Long-term stability data

High Concentration (ng/ml)			Low Concentration (ng/ml)		
Nominal	Measured (-20 °C)	Measured (-70 °C)	Nominal	Measured (-20 °C)	Measured (-70 °C)
45.74	47.50	45.36	11.40	12.42	11.08
45.74	46.00	48.79	11.40	11.57	11.66
45.74	47.19	48.97	11.40	11.74	12.10
45.74	45.93	47.00	11.40	11.74	11.98
45.74	46.83	47.70	11.40	11.52	11.75
Mean	47	48	**Mean**	11.8	11.7
Std Dev	0.7	1.5	**Std Dev**	0.4	0.4
% nom	102.1	104.0	**% nom**	103.5	102.8
CV %	1.5	3.1	**CV %**	3.1	3.4

By comparing the measured concentrations against the nominal concentrations given in Table-55 a correlation coefficient of 1.02 at -20°C, and 1.04 at -70°C were obtained, indicating that there was no significant degradation of fentanyl during the period of 100 days (see Figure-61).



Slope (-20°C) =	1.02
Slope (-70°C) =	1.04

Figure - 61: **Long-term stability chart**

8.9. Specificity

No interfering or late eluting peaks were found in the six blank plasma extract chromatograms obtained from six different sources of plasma. Figure-62 is an example of a blank plasma extract chromatogram, and Figure-63 is the chromatogram of an extract at LLOQ (0.30ng/ml). By comparing these two chromatograms it is clearly indicated that the method was specific and selective for fentanyl without any interfering peaks.

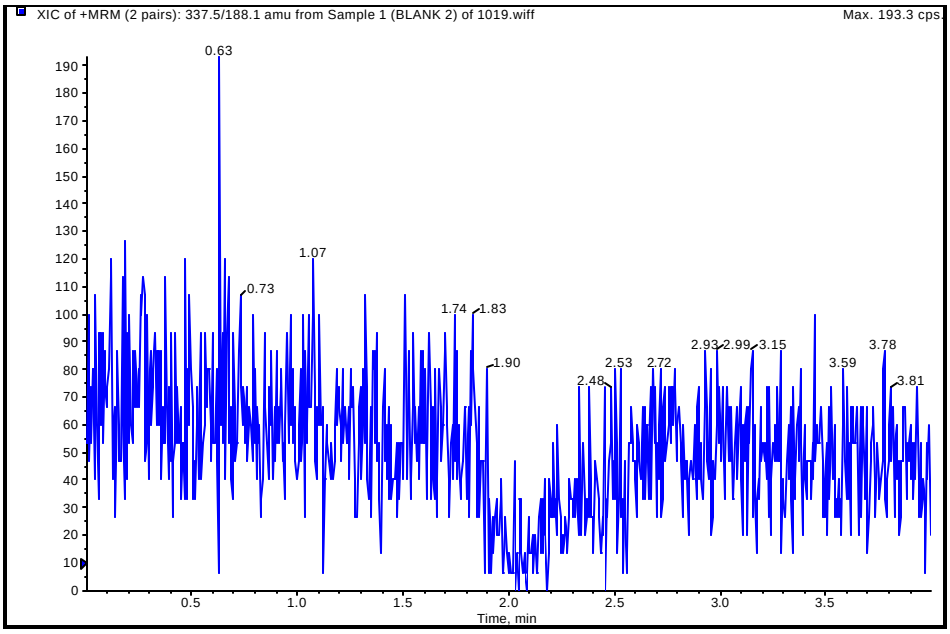


Figure -62: Chromatogram of a blank plasma extract

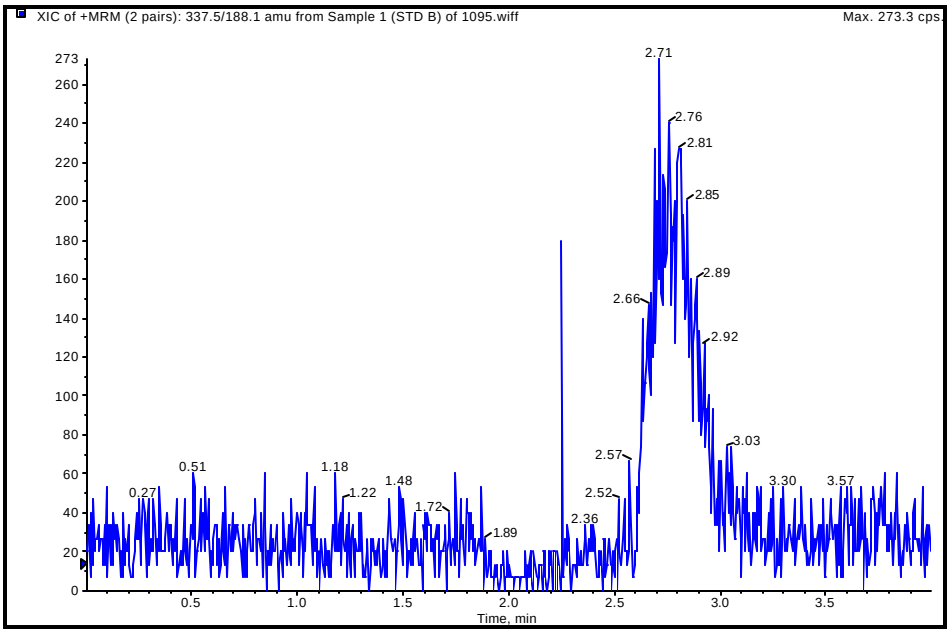


Figure -63: Chromatogram of plasma extract at LLOQ (0.30 ng/ml)

8.10. Sensitivity

The LLOQ, defined as that concentration of fentanyl which can still be determined with acceptable precision (CV % < 20) and accuracy (bias < 20 %), was found to be 0.30 ng/ml with a signal–to–noise ratio of ~18 (Figure-64).

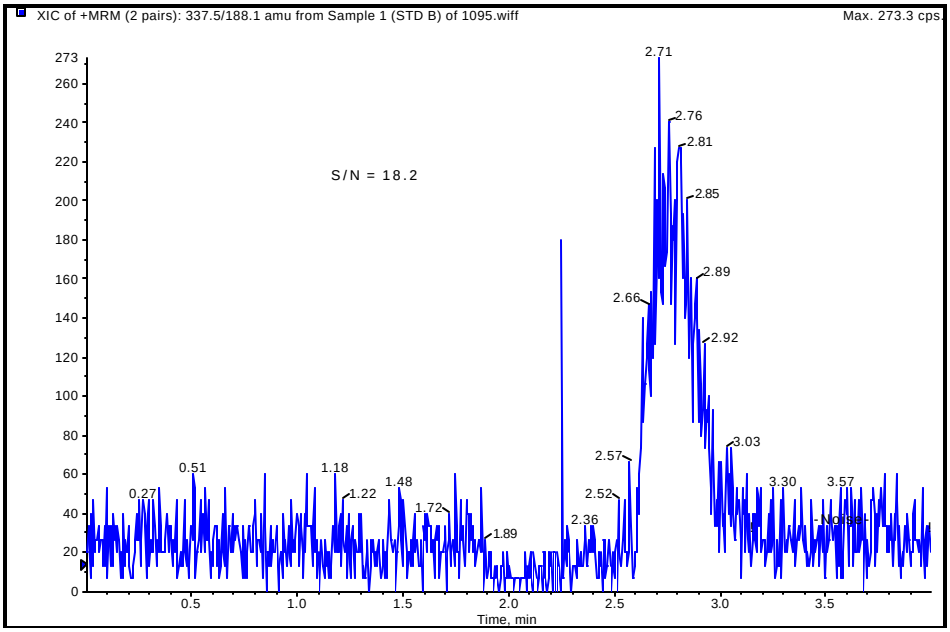


Figure -64: Signal–to–Noise ratio at LLOQ (0.30 ng/ml).

8.11. Recovery

Absolute recovery of a bioanalytical method is the measured response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of the analyte that is present in the sample (Bressolle, 1996)

$$\text{Absolute recovery} = \left(\frac{response\ of\ spiked\ plasma\ (processed)}{response\ of\ standard\ solution\ (unprocessed)} \right) \text{x } 100$$

Peak areas of three different quality control concentrations and peak areas obtained from the SPVS are used in calculating the recovery of the analyte according to the above-mentioned formula. Absolute recoveries of the analyte were determined in triplicate at high, medium, and low concentrations of the analytes in plasma and are summarized in Table-56a to 56c.

Table -56a: Intra-day absolute recovery of analyte using response factor areas

SAMPLE	Analyte concentration ng/ml	MEAN OF PEAK AREAS		Absolute Recovery (%)	CV (%)
		After Extraction	SPVS Values		
High Conc.	65.7	424283	753097	56.3	21.19
Medium Conc.	16.4	115600	187988	61.5	4.93
Low Conc.	3.16	21805	36222	60.2	8.93

Table -56b: Inter-day 1 absolute recovery of analyte using response factor areas

SAMPLE	Analyte concentration ng/ml	MEAN OF PEAK AREAS		Absolute Recovery (%)	CV (%)
		After Extraction	SPVS Values		
High Conc.	65.7	485450	704479	68.9	15.27
Medium Conc.	8.21	67420	88033	76.6	12.32
Low Conc.	0.797	7239	8546	84.7	10.98

Table -56c: Inter-day 2 absolute recovery of analyte using response factor areas

SAMPLE	Analyte concentration ng/ml	MEAN OF PEAK AREAS		Absolute Recovery (%)	CV (%)
		After Extraction	SPVS Values		
High Conc.	65.7	399500	610309	65.5	10.77
Medium Conc.	8.21	61652	76265	80.8	11.37
Low Conc.	0.797	6772	7404	91.5	11.72

It was difficult to determine the recovery with accuracy and precision because of the apparent drifting sensitivity of the detector resulting from the latent matrix effect. However, the tendency of the recovery to increase with decreasing plasma concentration extracts appears real and cannot be explained at this stage. For this reason, the recovery was determined in all three validation batches and this tendency shown to be consistent.

Representative chromatograms of the plasma standards and QCs at high, medium and lower fentanyl concentration, and of a blank plasma are presented below.

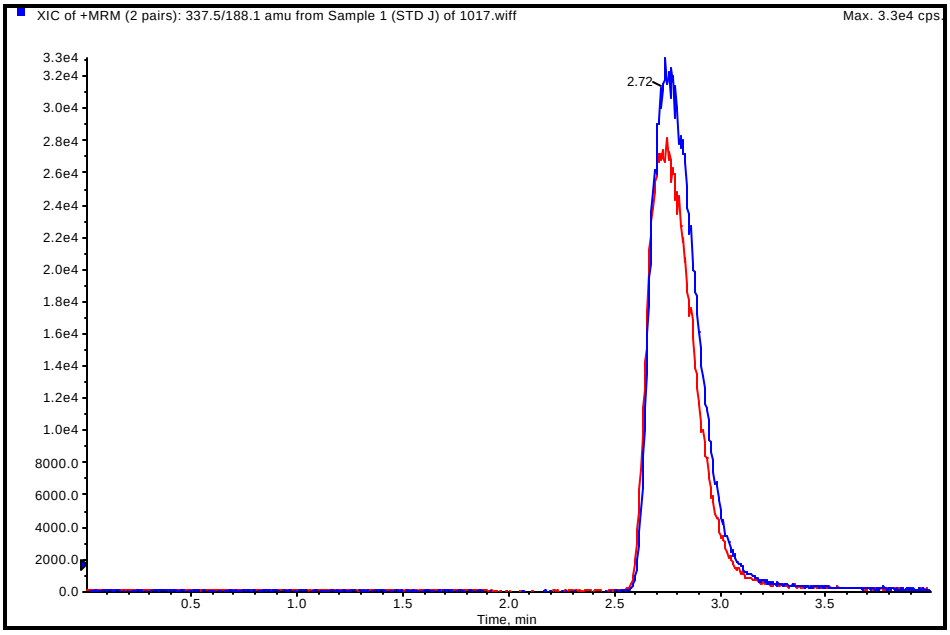


Figure -65: Chromatogram of an extract of STD J (77.6 ng/ml)

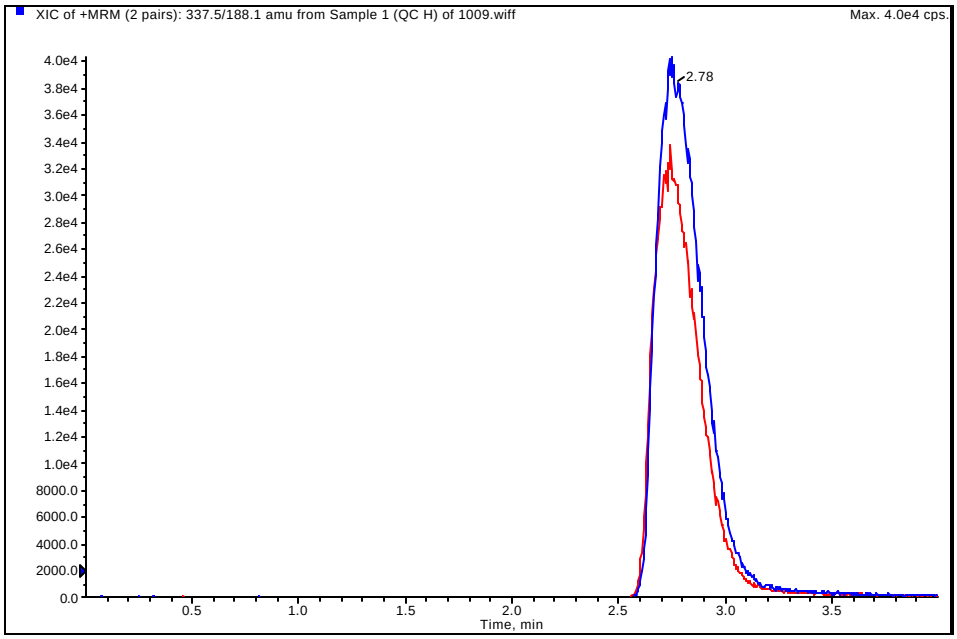


Figure -66: Chromatogram of an extract of QC H (65.7 ng/ml)

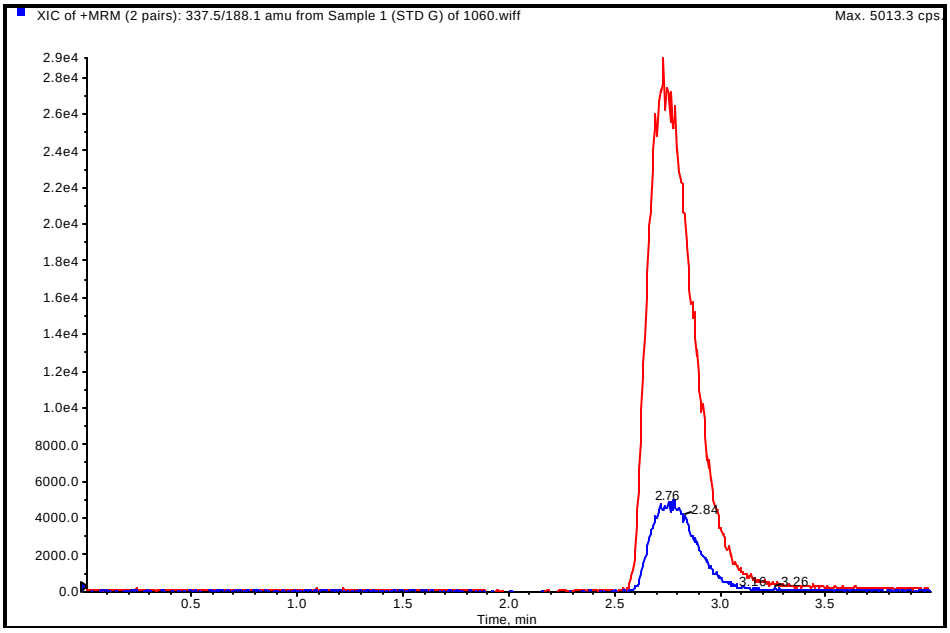


Figure -67: Chromatogram of an extract of STD G (9.7 ng/ml)

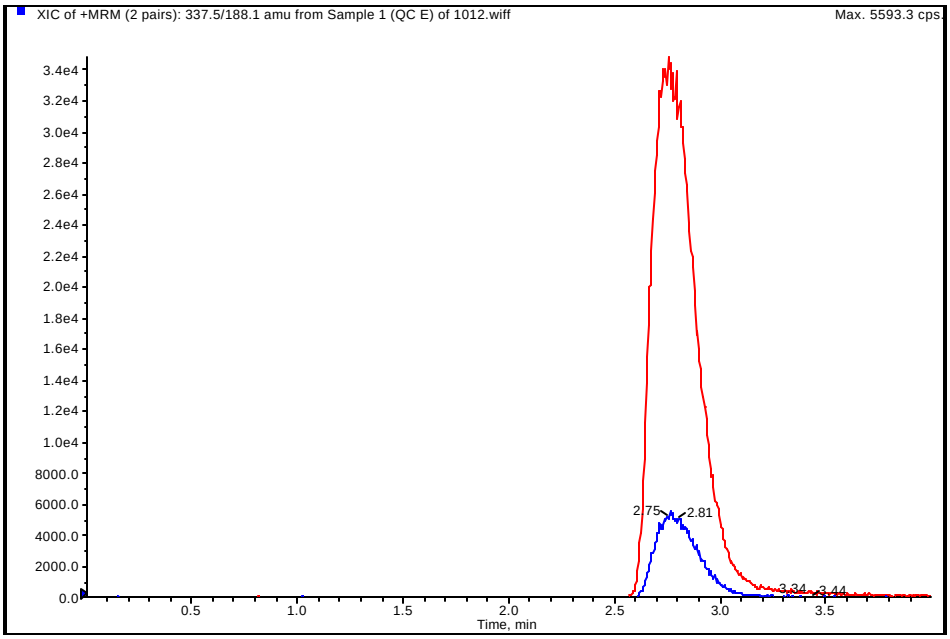


Figure -68: Chromatogram of an extract of QC E (8.21 ng/ml)

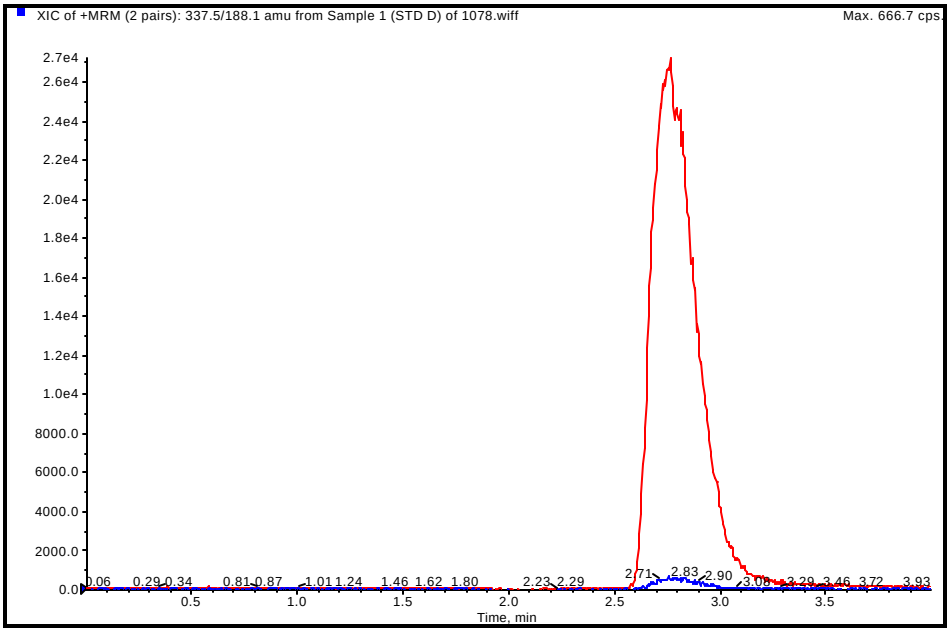


Figure -69: Chromatogram of an extract of STD D (1.21 ng/ml)

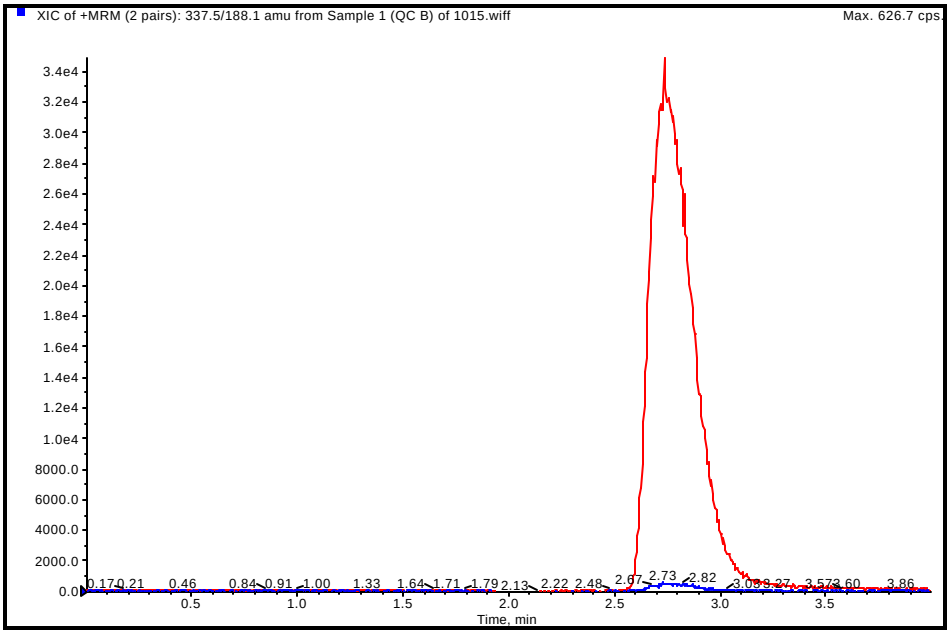


Figure -70: Chromatogram of an extract of QC B (1.59 ng/ml)

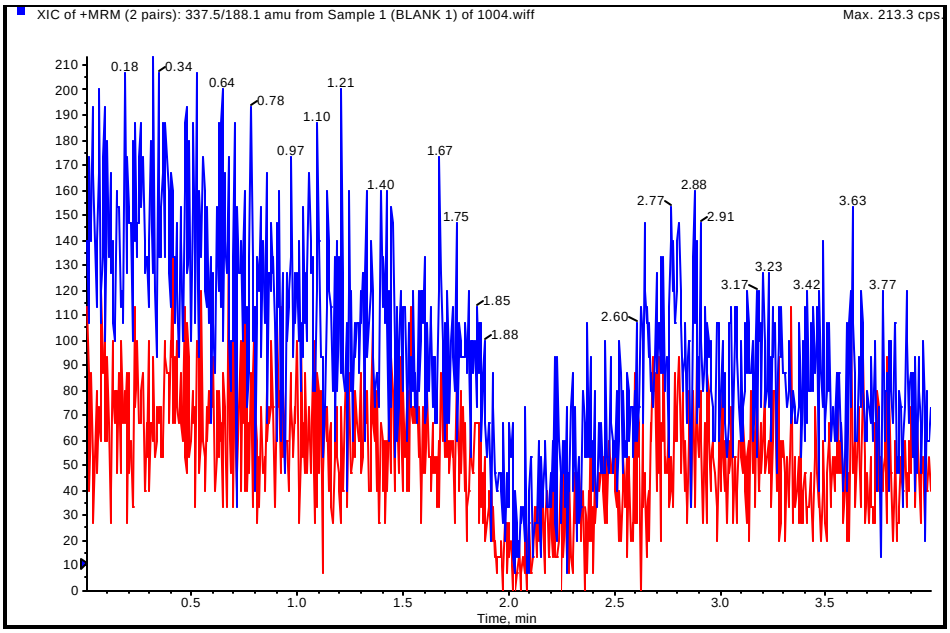


Figure -71: Chromatogram of an extract of blank plasma

8.12. Conclusion

A sensitive, rugged and highly automated LC-MS/MS method has been developed for the determination of the opioid analgesic fentanyl in human plasma with a PE SCIEX API 2000 triple quadruple mass spectrometer in Multiple Reaction Monitoring (MRM) and positive ion mode, using TurboIonSpray ionisation. Sensitivity in sub-ng/ml range was obtained through the use of an extremely sensitive and selective MS/MS detector combined with an efficient extraction technique. The turn-around time on the instrument was short, and a sample could be injected every 5 minutes onto the LC-MS/MS system. The use of a stable isotope-labelled internal standard made it a robust assay method in spite of what appeared to be a significant latent matrix effect, and a series of 100 samples could be injected without any loss of the instrument performance. The intra- and inter-batch assay precision as determined from quality control samples at 6 levels analyzed during validation of the method were in the range of CV = 2 – 7 %. The lower limit of quantification of 0.30ng/ml (CV % ~9) is a bit disappointing but can probably be improved upon by tweaking the acquisition parameters manually instead of using the auto tune function.

9 GC/MS ASSAY METHOD DEVELOPMENT

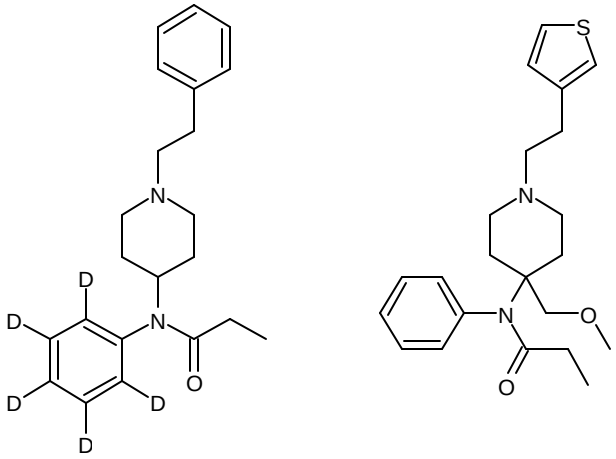
9.1 Assay Procedure

The initial assay method development process on the GC/NPD system was undertaken to obtain leads about the retention times of the analyte and internal standard, reproducibility, sensitivity and extraction efficiency of the method under consideration. Therefore, for the GC/MS method development they were now used as starting points to which some modifications could be made.

9.1.1 Preparation of Stock Solutions

Materials

The fentanyl and papaverine stock solutions and injection solutions (in toluene for injection onto the GC column) prepared during the preliminary GC/NPD method development were used as such. In addition, stock solutions and injection solutions (for injection onto the GC column) of sufentanil were prepared at relevant concentrations in the same manner as described for fentanyl since the sufentanil used was also obtained as a solution for injection (containing 5 µg/ml sufentanil free base, Janssen Pharmaceutica). D_5-fentanyl was obtained from Cerilliant^{TM} , Austin, Texas in a 1 ml amber sealed glass ampoule containing 100 µg/ml D_5-fentanyl in methanol. Stock solutions at relevant concentrations of D_5-fentanyl were obtained by spiking this methanol solution into the respective solvents. High purity solvents (Burdick & Jackson), such as toluene, ethyl ether, n-butyl chloride, were used as received.



a) D_5-Fentanyl **b) Sufentanil**

Figure -72: Chemical structure of a) D_5-Fentanyl and b) Sufentanil

9.1.2 Instrument and Chromatographic Conditions

The analyses were carried out on a Hewlett Packard 6890 series GC system equipped with a 7683 autosampler and a 5973 series mass selective detector (MSD) in EI mode (70 ev). High purity helium was used as the carrier gas at a constant flow rate of 2.5 ml/min. The column was the same capillary column (30 m x 0.32 mm i.d. and a film thickness of 0.25 μm) which was used in the GC/NPD method described in section 5.1.2.

GC-conditions

The following preliminary GC conditions were used to obtain information on retention times of fentanyl, papaverine and sufentanil on the GC/MS instrument set up.

- The injector temperature was set at 280°C.
- The oven temperature was programmed as follows: initially 220°C for 1min. then raised to 300°C at 20°C/min. and held for 3 min.
- Equilibration time = 0.5 min.
- A constant flow mode of 2.5 ml/min was used for He (column carrier gas).
- Total flow = 15.9 ml/min.
- Average velocity = 62 cm/min.

- Injection pulse = 36.0 p.s.i. for 1 min.
- Split ratio = 4:1 Split flow =10 ml/min.
- Gas saver = 20 ml/min. 2 minutes after injection.

MS-conditions

- EM voltage = 1871 V
- Solvent delay = 3 min.
- MS zone temperatures: MS source = 230°C, MS quad = 150°C
- Detector setting: full-scan m/z 50 – 800 in positive ion scan mode.
- Volume injected = 3 µl.

Solutions of fentanyl (5 µg/ml), papaverine (5 µg/ml) and D_5-fentanyl (1 µg/ml) in toluene were injected and chromatographed to obtain their respective mass spectra, which are presented below.

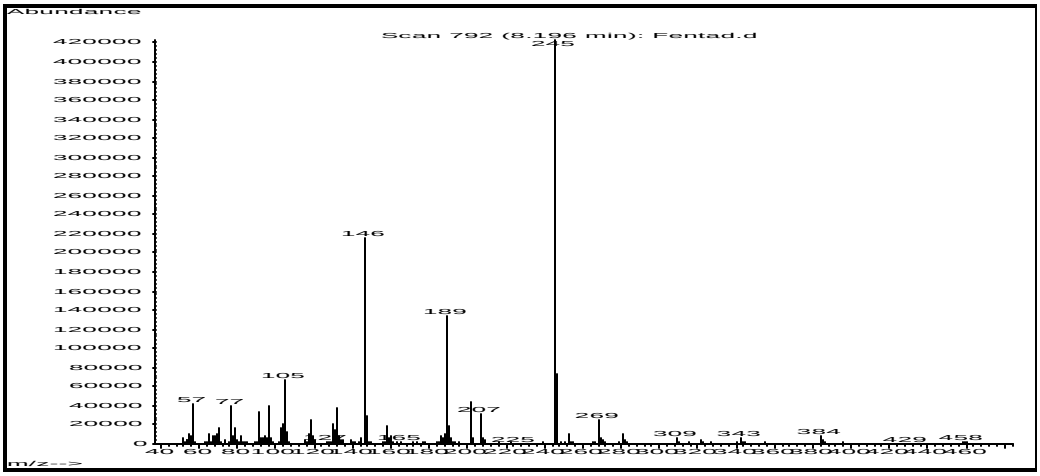


Figure -73: Mass spectrum of 5 µg/ml of fentanyl in toluene.

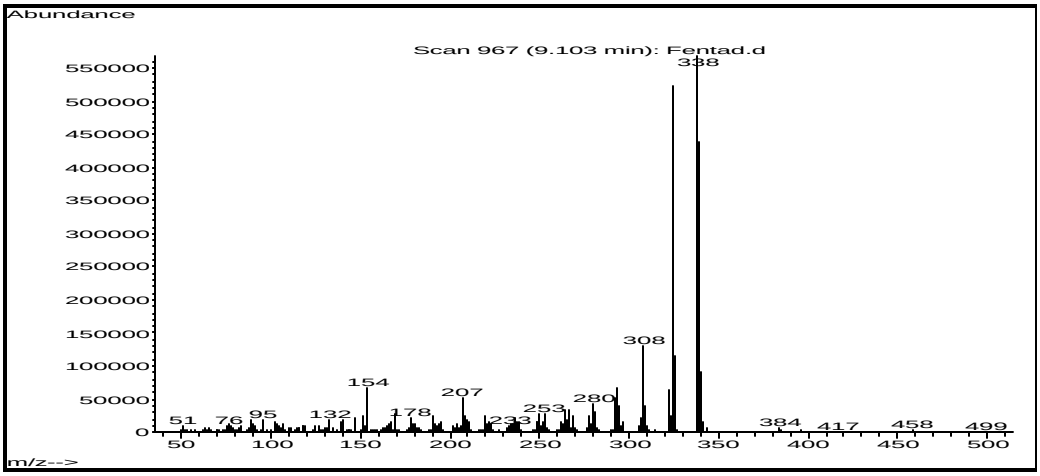


Figure -74: Mass spectrum of 5 mg/ml of Papaverine in toluene.

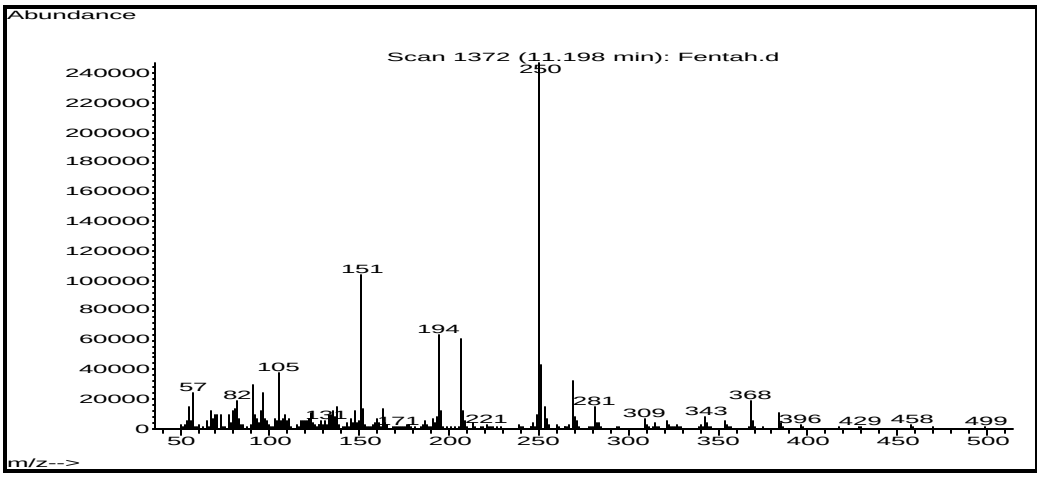


Figure -75: Mass spectrum of 1 mg/ml of D_5-fentanyl in toluene.

9.1.3 Chromatographic Results

Using the GC/MS conditions stated in 9.1.2, a chromatographic run for D_5-fentanyl (100 ng/ml) was done. A peak at 5.74 min was obtained (volume injected was 3 µl).

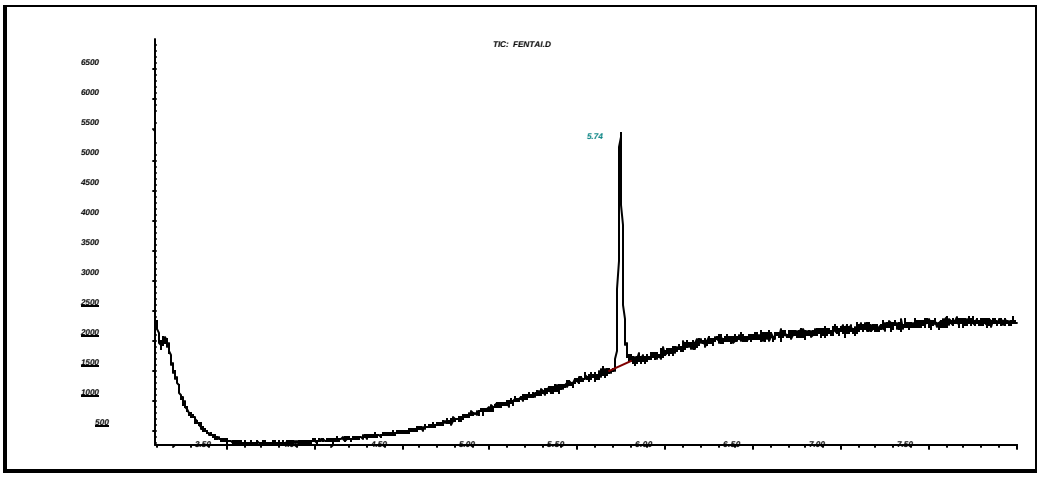


Figure -76: Chromatogram of 100 ng/ml D_5-fentanyl in toluene (SIM m/z = 250)

To a previous reconstituted plasma extract (sec. 5.1.4) of Std-A (1000 ng/ml fentanyl containing the internal standard papaverine), 100 µl of a 100 ng/ml D_5-fentanyl in toluene solution were added as an external std. and a chromatogram (Figure-77) was obtained under the conditions described above.

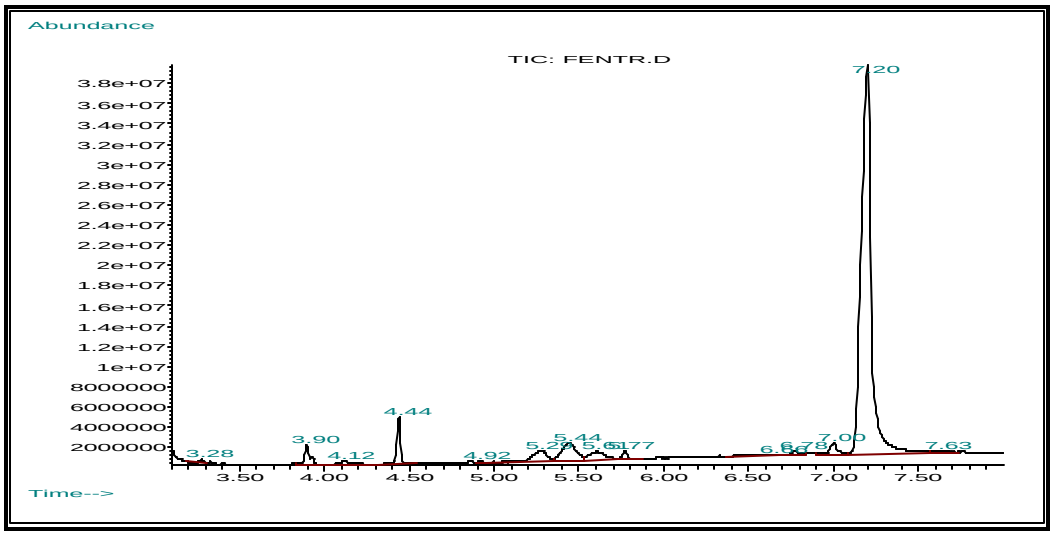


Figure -77: A full-scan (m/z = 50-800) chromatogram of a 1000 ng/ml plasma-fentanyl extract containing papaverine and D_5-fentanyl as internal and external stds. respectively

Due to the very strong peak with t_R = 7.20 min. the fentanyl (including overlapping D_5-fentanyl) and papaverine peaks were identified as the barely visible peaks with t_R = 5.77 min. and 6.75 min. by obtaining the following extracted ion chromatogram (m/z = 245,250,338) from the chromatogram in Figure-77.

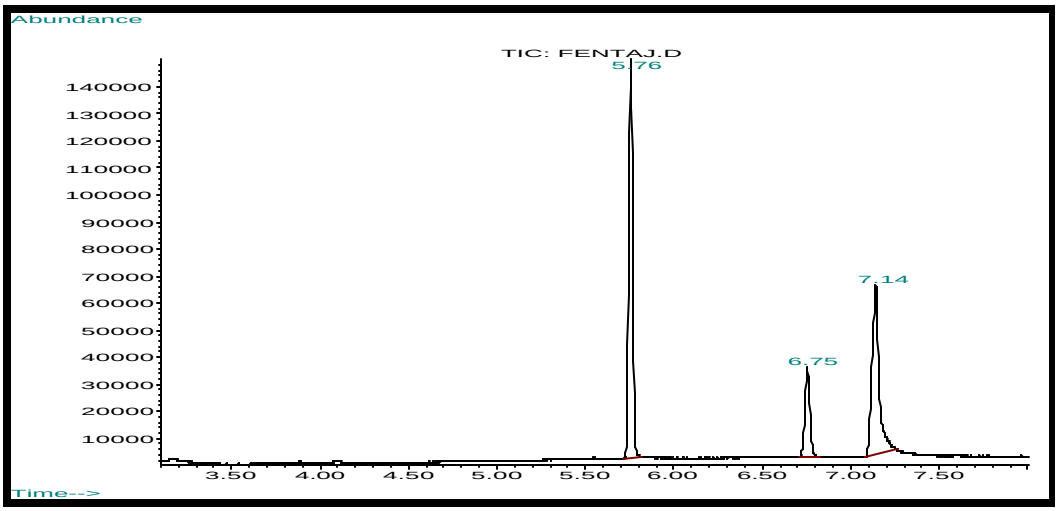


Figure -78: Extracted ion chromatogram of 1000 ng/ml plasma-fentanyl extract containing papaverine and D_5-fentanyl as internal and external standards respectively. Extracted ions m/z = 245, 250 and 338

Although much smaller, the peak at t_R = 7.14 min. was still clearly visible and had to be identified if possible. An extracted ion chromatogram (m/z = 245 and 250) indicated that the peak with t_R = 7.14 min. was shown to arise through detection of the ion with m/z = 245 as shown in the following chromatogram.

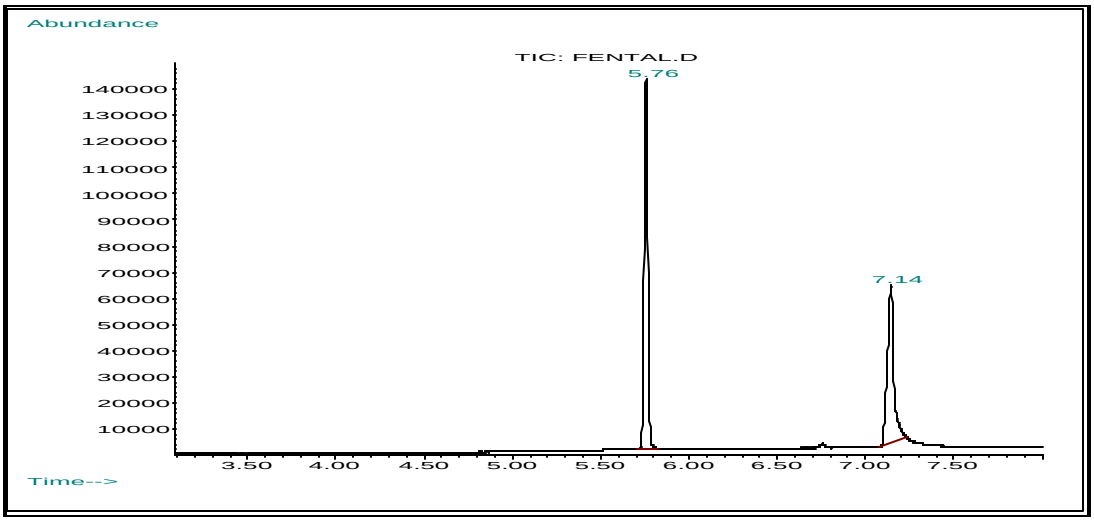


Figure -79: Extracted ion chromatogram of 1000 ng/ml plasma-fentanyl extract containing papaverine and D_5-fentanyl. Extracted ions m/z = 245 and 250.

A mass spectrum of the peak with t_R = 7.14 obtained from the chromatogram in Figure-77 aided in the identification of the component as Cholesterol.

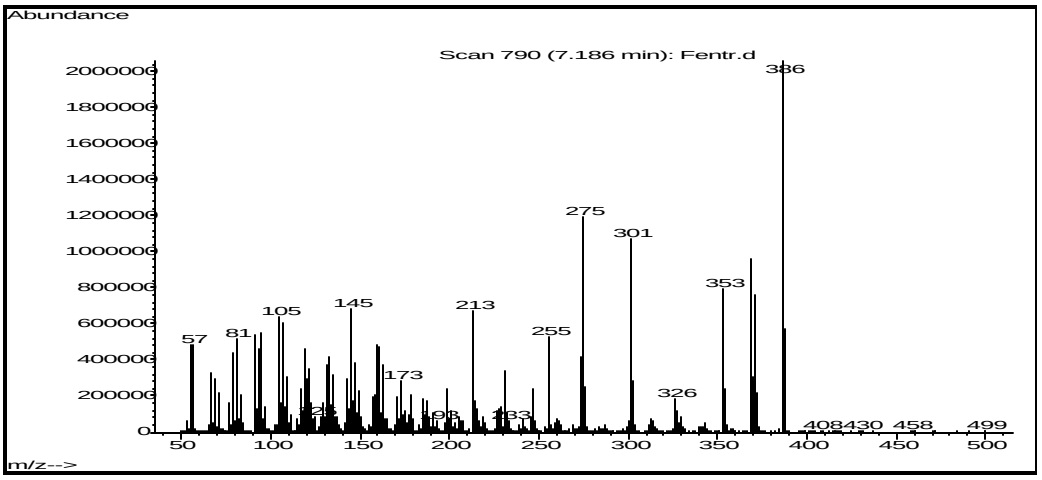


Figure -80: Mass spectrum of component with retention time = 7.14 min. (Cholesterol)

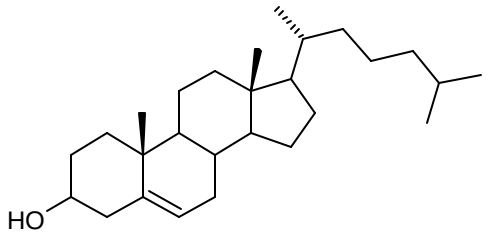


Figure -81: Chemical structure of cholesterol

Although the mass spectrum of Cholesterol contains a fragment ion with m/z = 245 at very low abundance, the concentration of cholesterol in plasma is high enough relative to the concentrations of fentanyl after therapeutic doses, to produce peaks of comparable size in the chromatograms of "dirty" plasma extracts, i.e. extracts not cleaned up by back-extraction to obtain mainly basic components. Nevertheless, it was decided to assess the simple extraction procedure further by testing its reproducibility and also investigating alternative extraction solvents.

9.1.4 Assessment of the Ether Extraction Procedure

To check the reproducibility of the ether extraction method a 5 ng/ml fentanyl-plasma solution was prepared by spiking 20 ml of plasma with 2 µl of fentanyl citrate injection solution (50 µg/ml fentanyl base equivalent) and 1 ml aliquots of the plasma extracted in six-fold as follows:

- 1 ml Plasma sample in 5 ml disposable glass ampoule
- Add 100 µl of a internal standard solution (100 ng/ml D_5-fentanyl in 0.01 N HCl) , 100 µl 4 M NaOH and 2 ml ethyl ether.
- Vortex mix for 2 min.
- Centrifuge at 2000 rpm for 5 min.
- Freeze aqueous phase in a freezing bath at ~ - 20 °C
- Decant organic layer into another ampoule and evaporate it at 40°C under a N_2 stream
- Reconstitute the residue in 100 µl of toluene
- Transfer to a 200 µl autosampler injection vial and inject 3 µl onto the GC-column.

MS Conditions

The MS conditions were the same as those used before, except that the mass spectrometer was operated in the selected ion monitoring (SIM) mode to detect ions with m/z = 245 and 250 only. The dwell time was set at 30 . for each ion. The results of this experiment are summarised in Table-57.

Table-57: Reproducibility data of 5ng/ml plasma-fentanyl extracts (n=6).

Injection #	Fentanyl peak area	D_5-fentanyl peak area	Peak area ratio
1	94836	517900	5.5
2	90117	405356	4.5
3	74473	398044	5.3
4	88533	435349	5.0
5	97416	441822	4.5
6	80568	405103	5.0
Mean:	**87657.17**	**433929**	**0.20**
CV%	**9.91**	**10.33**	**8.09**

Although the assay method appeared to be fairly reproducible, the relatively high CV of the peak area ratio was disconcerting in view of the fact that an isotope-labelled internal standard was being used.

Alternative extraction solvents, toluene and n-butyl-chloride, were tested. In three different 10 ml ampoules 100 µl of fentanyl citrate (50 µg/ml fentanyl equivalent injection solution) and 100 µl of 1 M NaOH were pipetted. Then 5 ml of toluene to the first ampoule, 5 ml of ethyl ether to the second, and 5 ml of n-butyl chloride to the third were added and extracted. After decantation, the organic phase was evaporated, the extracts reconstituted in 100 µl toluene and 3 µl injected onto the column.

The results obtained are presented in Table-58.

Table-58: Comparison of extraction efficiency of different organic solvents

Extraction solvent used	Peak height	Peak area
Toluene	124652	2281490
Ethyl ether	137215	2783040
n-Butyl chloride	129198	2445734

Although the extraction with ether appears marginally better than with toluene and n-butyl chloride the results indicate that any of the above solvents could be used as extraction solvent if a choice of solvent had to be made for some specific reason. It was decided to develop the assay method further using ether as the extraction solvent.

Recovery assessment

Stock solutions of fentanyl and plasma extracts of fentanyl with internal standard were prepared.

10 µl of fentanyl citrate (50 µg/ml fentanyl equivalent) + 10 µl of 10 M NaOH+100 µl of 100 ng/ml D_5-fentanyl + 5 ml ethyl ether were pipetted in a 10 ml ampoule and vortex mixed. Then after centrifugation for 10 min. 2 ml of the upper layer was transferred into another ampoule and evaporated at 40°C under N_2 gas. The residue was reconstituted in 100 µl of toluene to get a final concentration of 2000 ng/ml.

Everything being the same as above, the ether layer (5 ml) was aspirated and mixed with1ml of plasma alkalinised with 200 µl of 10 M NaOH. After vortex mixing and centrifugation, 2 ml of the upper layer was transferred into another ampoule and evaporated. The residue was reconstituted in 100 µl of toluene to obtain 2000 ng/ml fentanyl.

Table-59: Summary of recovery data.

Fentanyl conc.	ISTD conc.	Fentanyl Peak Area	ISTD Peak Area		% recovery
2000ng/ml(stock)	100 ng/ml	2117877	36345		100%
2000 ng/ml(extract)	100 ng/ml	1717492	75368		81.1%

On-instrument Reproducibility

During the injection of the extracts, fluctuations in peak areas for repeatedly injected samples of the same concentration from the same vial were observed. Therefore, to check the on-instrument reproducibility a 200 ng/ml fentanyl-plasma was extracted and the same extract injected 20 times. As can be seen in Figure-82 the variability of the peak area of fentanyl was considerable. The steady decrease of the peak area response after the initial five injections can probably be attributed to fouling of the ionisation source as the extracts were not "clean." There was a great difference in peak area between the first and last injections, 21805 and 8620 respectively.

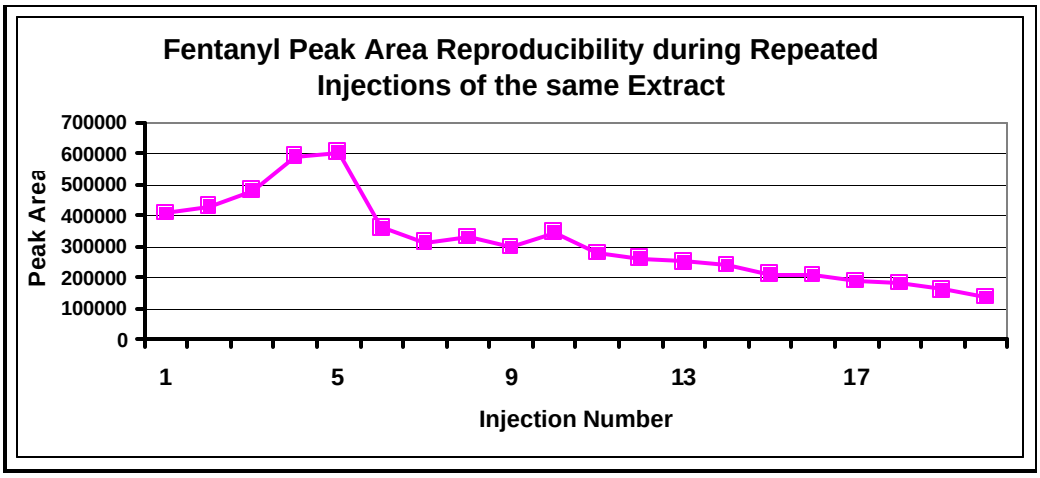


Figure -82: Peak area reproducibility chart-1

To assess whether changing the chromatography conditions would have an influence on this downward trend another 100 ng/ml plasma extract of fentanyl was prepared containing D_5-fentanyl as internal standard. The chromatography conditions were changed as follows:

Initial oven temperature was lowered to 100°C from 240°C, and held for 1 min., then raised to 300°C at 30°C/min. and held for 2 min.

- The inlet temperature was lowered from 280°C to 240°C
- Solvent delay time was raised to 8 min.
- The ion dwell time was also raised from 30 ms. to 50 ms.

Thirteen injections of the extract were made and the results are presented in Figure-83.

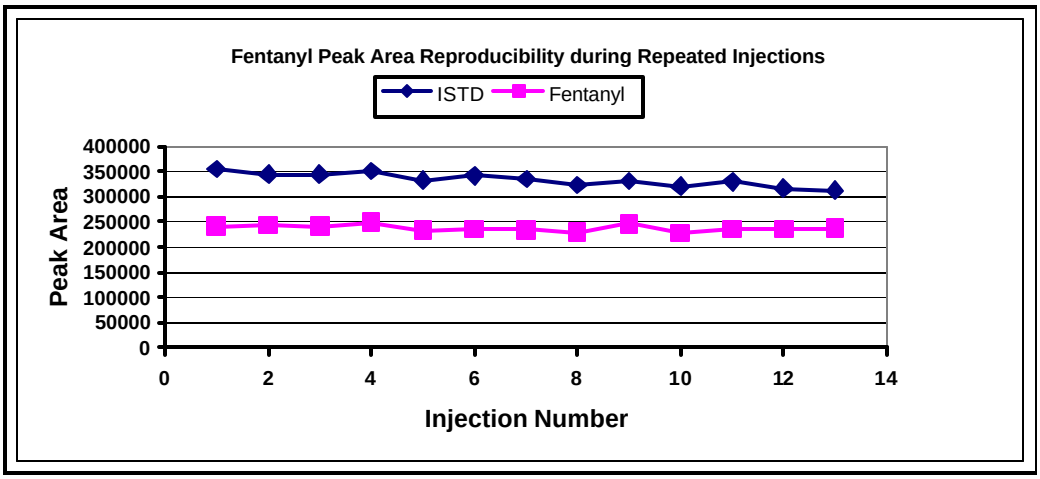


Figure -83: Peak area reproducibility chart-2

9.1.5 Optimisation with clean extracts and an alternative column

The downward trend in peak area was less steep but still noticeable. Since it was difficult to rationalise how changing the chromatography conditions could have resulted in this improvement, it was decided to improve the purity of the extracts even if it meant that the time spent on sample preparation would increase considerably.

The scarcity, difficult availability and the cost of D_5-fentanyl also prompted one to revert to sufentanil as an alternative internal standard. As the injection of a large number of "dirty" extracts had taken its toll of the column, it was also decided to replace the column with a CP-SIL 8 CB fused silica column which was available off the shelf.

Assay method development described in section 5 was now first performed on the GC/NPD system before reverting back to the GC/MS assay method development. The mass spectrometer's ion source, and septum purge and split vent flow valves were cleaned, and performance verification assessed with methyl stearate (1 ng/µl) to check the reproducibility and sensitivity of the GC/MS. Then a method (EFR.M) was loaded with the following conditions:

GC-conditions:

- Inlet temperature 250°C,
- Injection 3 µl pulsed splitless
- Pressure variable since constant flow mode used
- Injection pulse pressure 36 psi for 1 minute
- Purge flow to split vent 4.9 ml/min.
- EM voltage 1776 V
- Solvent delay 4min. Gas saver = 20 ml/min. after 2 min.
- Oven temperature Initial 80°C for 1min., then raised to 300°C at 30°C/min. and held for 2 min. (total run time 10.33min.).
- Transfer line temperature 280°C

MS-conditions:

- MS-Quad 150°C;

- MS-source 230°C

Using the above method, a sequence of 50 injections from the same vial containing 500 ng/ml fentanyl in toluene was run to check the on-instrument reproducibility. There was a steady downward tendency in peak area (see Figure-84). The mean height = 35486.26; % CV 6.53 and mean area = 478235.5; %CV 6.46.

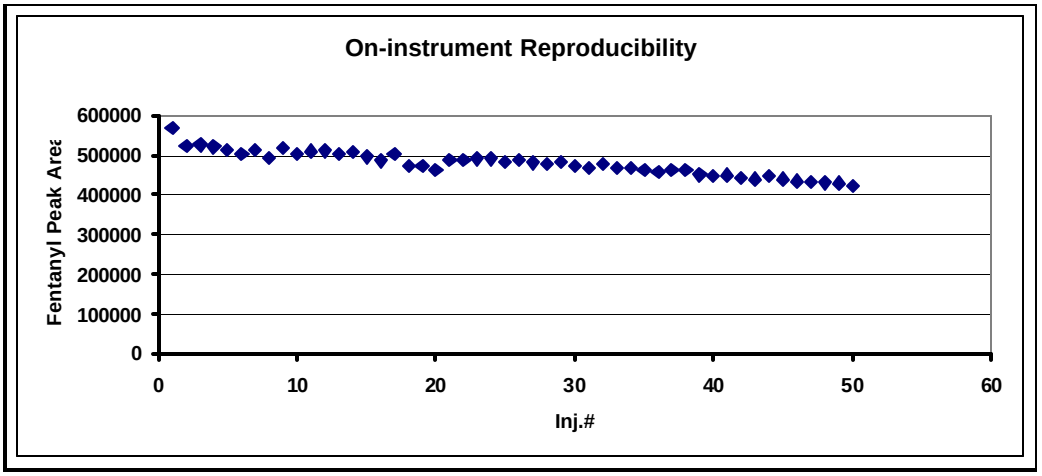


Figure -84: On-instrument reproducibility chart-3

To optimize the reproducibility several injections of the 500 ng/ml fentanyl solution in toluene under different GC & MS conditions were performed (see Table -60).

Table -60: Summary of chromatographic runs under different GC & MS conditions (all temperatures are in ^{o}C and time in minutes).

Injection #	Oven temperature program				Inlet temp	MSD transfer line	RT (min)	Peak Height	Peak Area
	Initial temperature	Rate, ^{o}C/min	Final temp	Final time					
1	150 for 1min	NA	285	12.7	280	280	8.18	20845	401676
	Level-1 240	50							
	Level-2 285	10							
2	85 for 0.75min	NA	315	0.75	270	270	11.18	51240	539542
	Level-1 315	22.5							
3	100 for 1min	NA	280	10	280	290	NA	NA	NA
	Level-1 280	70							
4	90 for 1min	NA	280	0	260	260	12.94	25623	647977
	Level-1 240	30							
	Level-2 280	5							
5	Isothermal 210	NA	NA	NA	260	210	NA	NA	NA
6	100 for 1min	NA	300	2	240	260	10.42	39316	618446
	Level-1 250	30							
	Level-2 300	10							
7	100 for 0.75min	NA	280	1	240	260	10.69	26671	600730
	Level-1 250	35							
	Level-2 280	5							
8	100 for 0.75min	NA	280	1	260	260	10.68	29340	651750
	Level-1 250	35							
	Level-2 280	5							

The last injection (# 8) gave the best results. Thus method EFR.M was modified in accordance to the conditions used in running injection number 8. Then to confirm the reproducibility, a 250 ng/ml fentanyl solution in toluene containing 250 ng/ml sufentanil as ISTD was injected 50 X from the same vial in sequence. The method was found to be reproducible with % CV 3.3 (see Table -61 and Figure -85).

Table-61: Summary of on-instrument reproducibility

(n = 50)	Fentanyl	Sufentanil	Ratio
Mean Height	12840.8	21334.22	0.6019
% CV	3.94	2.99	2.93
Mean Area	293109	508902.8	0.5759
% CV	3.31	2.49	1.38

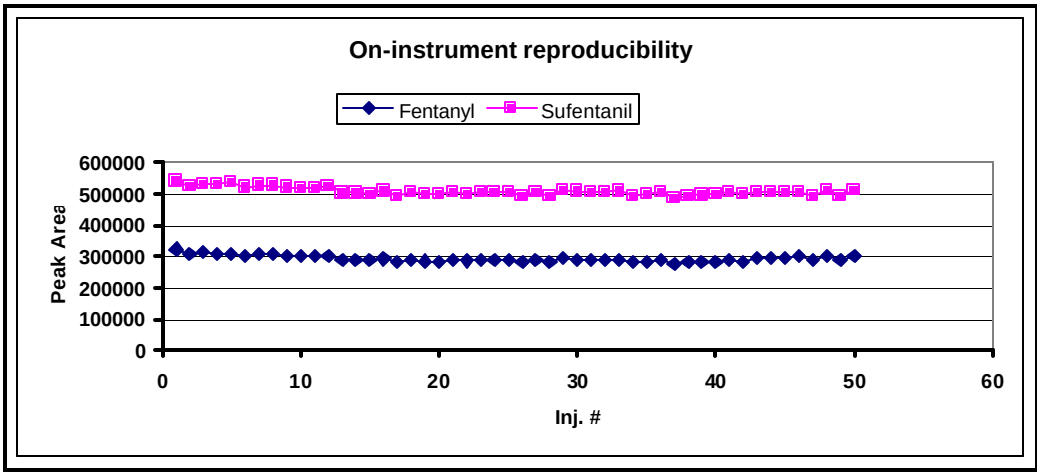


Figure-85: On-instrument reproducibility chart-4

9.1.6 Preparation of calibration standards

10 ml of plasma was spiked with 20 µl of fentanyl citrate injection solution (50 µg/ml fentanyl equivalent) to obtain 100 ng/ml fentanyl-plasma standard (STD A), which was diluted (1:1) with blank plasma up to 0.0475 ng/ml (STD L).

Aliquots (1 ml) of each standard were pipetted in to 10 ml ampoules to which 5 µl of sufentanil citrate injection solution (5 µg/ml sufentanil equivalent) was added to obtain 250 ng/ml sufentanil as ISTD. Then 100 µl of 10 M NaOH was added and extracted with 5 ml ethyl ether followed by back extraction with 3 ml of 1 N H_2SO_4 and re-extraction with 5 ml ethyl ether. The ether layer was decanted into another test tube and evaporated at 40°C. The residue was reconstituted with 100 µl of toluene and transferred into a 200 µl injection vial inserts. Finally 3 µl of the extracts were injected into the GC-column in sequence. The results obtained are summarised in Table-62.

Table-62: Fentanyl calibration standard data

		Peak Height			Peak Area				
Code	**Conc. (ng/ml)**	**Fentanyl**	**Sufentanil**	**Ratio**	**Fentanyl**	**Sufentanil**	**Ratio**	**%Recovery**	**S/N**
STD A	100	37056	15148	2.446264	883834	361867	2.442428	61.10	
STD B	50	20507	17198	1.192406	471043	417415	1.128476	72.60	
STD C	25	9202	15742	0.584551	218988	383536	0.570971	73.90	
STD D	12.5	4782	12469	0.383511	111558	315884	0.353161	69.88	
STD E	6.25	2579	13620	0.189354	62328	329235	0.189312	76.82	
STD F	3.125	1579	14564	0.108418	37170	357696	0.103915	86.10	
STD G	1.56	860	16692	0.051522	21523	402371	0.05349	97.85	
STD H	0.78	502	16674	0.030107	12481	409775	0.030458	100.00	
STD I	0.39	377	14788	0.025494	9801	362375	0.027047	89.90	12
STD J	0.19	243	15979	0.015207	6189	381797	0.01621	94.40	13
STD K	0.095	168	15673	0.010719	4676	384771	0.012153	95.140	5
STD L	0.0475	162	18895	0.008574	4034	466414	0.008649	100.00	6

Note: % recovery was calculated by comparing the extract's peak area with that of unextracted 250ng/ml fentanyl solution in toluene containing 250ng/ml sufentanil (ISTD).

The peak area ratios were plotted against fentanyl-plasma concentration (see Figure-86).

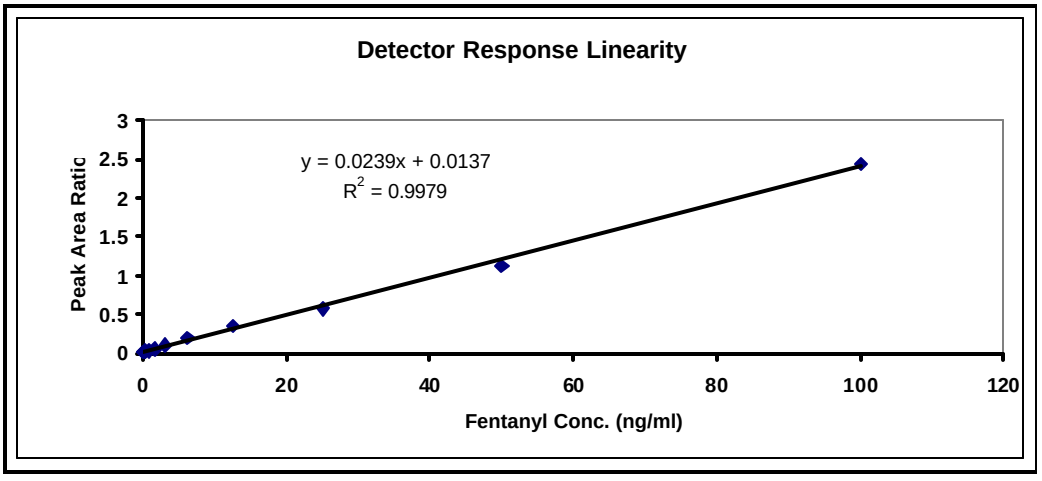


Figure-86: Calibration standard curve of fentanyl

Some representative chromatograms are given below.

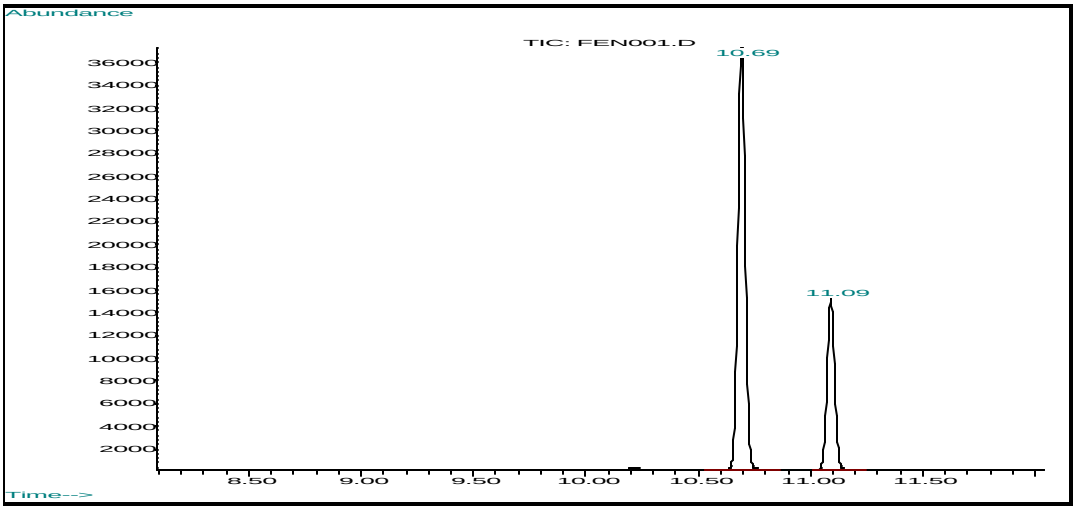


Figure -87: Chromatogram of 100 ng/ml plasma-fentanyl standard.

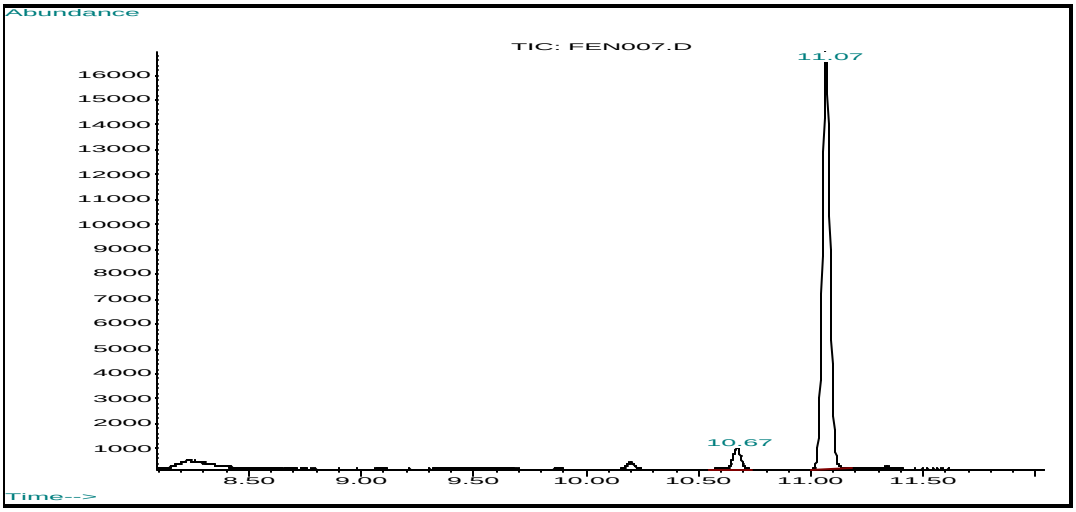


Figure -88: Chromatogram of 1.56 ng/ml plasma-fentanyl standard

A blank plasma extract was injected into the GC-column. The chromatogram obtained, even though the plasma was back-extracted with 1 N H_2SO_4, yielded a strong peak at 13.3 min. from cholesterol. In addition to this there was too much back ground noise, which makes quantitation at lower concentrations difficult.

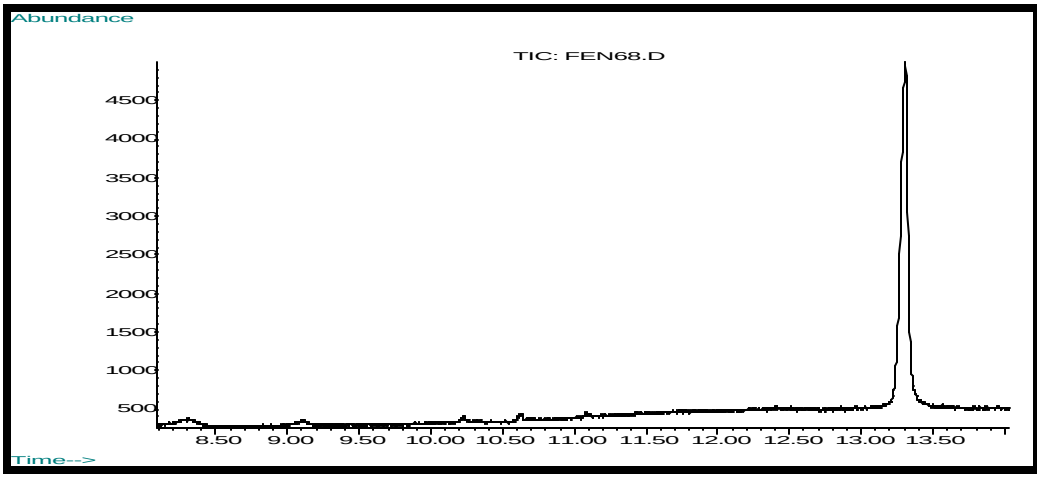


Figure -89: **Chromatogram of blank plasma extract.**

For further confirmation of the reproducibility of the method a 20 ng/ml fentanyl-plasma extract containing 5 ng/ml sufentanil (ISTD) was injected 12 x. Mean peak area, and CV % were calculated.

Table -63: Summary of reproducibility data.

Inj. #	Peak Area			Peak Area Ratio		
	Analyte	ISTD	Cholesterol	Analyte:ISTD	Analyte: Chol.	Chol.:ISTD
1	126177	57707	181907	2.1865	0.6936	3.1523
2	128412	59416	351388	2.1612	0.3654	5.9140
3	126806	46132	156523	2.7488	0.8101	3.3929
4	117603	44377	280015	2.6501	0.4200	6.3099
5	132159	50326	299815	2.6261	0.4408	5.9575
6	126304	46867	327704	2.6949	0.3854	6.9922
7	108162	35195	234271	3.0732	0.4617	6.6564
8	119530	50200	275571	2.3811	0.4338	5.4895
9	120718	44795	301655	2.6949	0.4002	6.7341
10	115412	49422	288116	2.335	0.4006	5.8297
11	117907	51636	258316	2.2834	0.4564	5.0026
12	114040	46461	135348	2.4545	0.8426	2.9132
MEAN	121102.5	48544.5	257552.42	2.5242	0.5092	5.3620
%CV	5.76	13.04	26.34	10.69	33.43	26.91

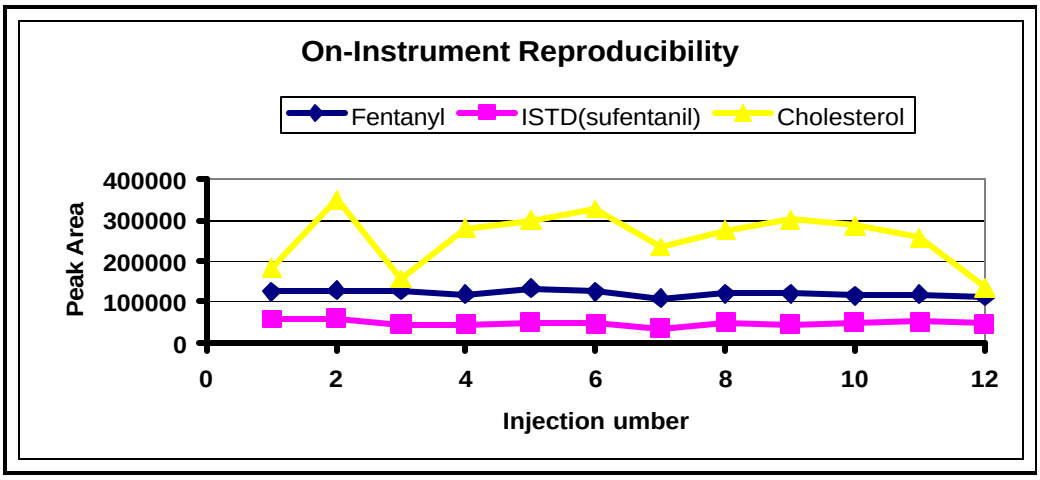


Figure -90: On-instrument reproducibility chart-5.

As indicated in the previous tables and charts, method EFR.M is reproducible. However the turn-around time (17 minutes) between consecutive injections in a sequence is high. Therefore to run a large sample batch will take a long time. Thus to shorten the run time, changes in the method such as increasing the initial oven temperature or the ramping rate of oven temperature, MSD transfer line temperature, etc. was investigated. However, with increase in initial oven temperature (>100°C) or ramping rate (>40°C/min), sensitivity and reproducibility of the method decreased significantly.

Due to the unavailability of the instrument, further development of the assay method was shelved. In retrospect it was a mistake to try to develop this assay method without extensive clean-up of the plasma extracts. However, the work done up to this point will be invaluable to anyone continuing with investigations to improve the assay method.

10. SUMMARY

The development and validation of bioanalytical assay methods suitable for the quantification of fentanyl in human plasma is discussed. A thorough literature survey was done, and few earlier works are summarized in chapter-4, Table-3. Special care was paid to chromatographic optimization, extraction procedures, detector selection and method validation. A short summary of these methods are given:

- A sensitive, selective and rapid method for the determination of fentanyl in human plasma was developed, using Gas Chromatography equipped with a Nitrogen/Phosphorus selective detector (NPD). The sample preparation involved pre-treatment of plasma with saturated sodium hydroxide (pH ~12) to denature protein, and then fentanyl and sufentanil (ISTD) were extracted with ethyl ether followed by back-extraction with 1N H_2SO_4 and re-extraction with ethyl ether after basifying it with 10M sodium hydroxide solution. The aqueous phase was frozen, and the organic phase decanted into another ampoule and evaporated at 40°C under N_2. The residues were reconstituted in toluene and 3µl of it injected into the GC-column. Chromatography was performed on a Hewlett Packard 5890 series II Gas Chromatograph equipped with an autosampler (HP 7673) and a Nitrogen/Phosphorus selective detector (NPD). A Chrompac Cp-Sil 8CB fused silica capillary column (30 m x 0.32 mm i.d. and 0.25 µm film thickness of 5 % phenyl, and 95 % dimethyl polysiloxane) was used with high purity helium as carrier gas at a column head pressure of 25 psi, and a flow rate which varies with temperature, such as at 140°C = 4.82 ml/min.; at 200°C = 3.81 ml/min. etc. There were no interfering or late eluting peaks, and resolution between analyte and internal

standard was obtained in a chromatographic run time of 5 min. The assay method was validated over a range of fentanyl-plasma concentrations between 0.19 – 102 ng/ml, where the LLOQ was set at 0.773 ng/ml. The mean recovery of fentanyl was ~120 % with a coefficient of variation ~6 %.

- A selective, sensitive and rapid Liquid Chromatography-tandem mass spectrometry method for the determination of fentanyl in human plasma was developed. An Applied Biosystem API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using Electro Spray Ionization (ESI) with positive ionization was used (molecular ion of fentanyl m/z 337.8 to the product ion m/z 188.15; molecular ion of d_5-fentanyl (ISTD) m/z 342.6 to the product ion m/z 188.15). The sample preparation for LC-MS/MS involved denaturing protein with saturated sodium hydroxide, and then extracting fentanyl & d_5-fentanyl with ethyl ether followed by back-extraction with 2 % formic acid. The aqueous phase was frozen, and the organic phase discarded. The aqueous phase (extracts) were evaporated at low temperature (~30^{o}C) under N_2 for 1 min. to remove any remaining ethyl ether followed by immediate vortexing for 30 sec, and 20 µl was injected into the column. Chromatography was performed on a Supelco Discovery® C_{18}, 5 µm, 150 x 2 mm column with a mobile phase consisting of 0.05M HAC : methanol (40:60 (v/v)) pH adjusted to 4.54 using a 25% liquid ammonia at a flow rate of 0.2 ml/min. The assay method has been validated over the concentration range 0.30 – 155 ng/ml fentanyl in human plasma, based on a 1 ml sample size. The mean recovery of fentanyl was ~60% in the intra-day validation with a lower limit of quantification set at 0.30 ng/ml. The coefficient of variation within a run and between run was ~ 10 %.

KEYWORDS

Method development, validation, Gas Chromatography, High-Performance Liquid Chromatography, Nitrogen/Phosphorus selective detector (NPD), mass spectrometer, fentanyl, d_5-fentanyl, sufentanil.

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